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(71) Applicant *(for all designated States except US)*: UNIVERSITY OF SASKATCHEWAN [CA/CA]; 120 Veterinary Road, Saskatoon, Saskatchewan S7N 5E3 (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: REDDY, Police, Seshidhar [CA/US]; Apartment #1, 722 Clopper Road, Gaithersburg, MD 20878 (US). TIKOO, Suresh, Kumar [CA/CA]; 302 102 Edinburgh Place, Saskatoon, Saskatchewan S7H 5J7 (CA). BABIUK, Lorne, A. [CA/CA]; 245 East Place, Saskatoon, Saskatchewan S7J 2Y1 (CA).

(74) Agents: BRENNAN, Sean, M. et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).

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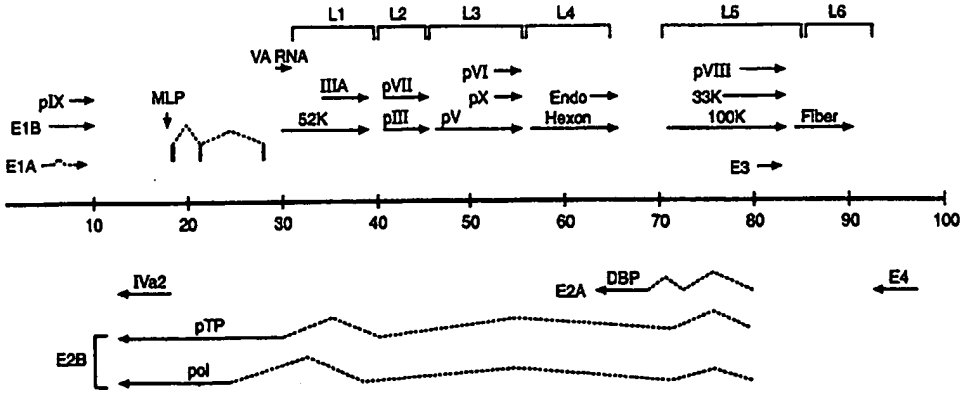

(57) Abstract

The complete nucleotide sequence of the genome of porcine adenovirus type 3 (PAV-3) is provided. Methods for construction of infectious PAV genomes by homologous recombination in procaryotic cells are provided. Recombinant PAV viruses are obtained by transfection of mammalian cells with recombinant PAV genomes. The PAV-3 genome can be used as a vector for the expression of heterologous nucleotide sequences, for example, for the preparation and administration of subunit vaccines to swine or other mammals. In addition, PAV-3 vectors can be used for gene therapy and expression of heterologous polypeptides. PAV-3 genome sequences can also be used for diagnostic purposes, to detect the presence of PAV-3 DNA in a subject or biological sample.

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PORCINE ADENOVIRUS TYPE 3 GENOME

TECHNICAL FIELD

The present invention is in the field of recombinant mammalian viral vectors. More particularly, it concerns recombinant porcine adenovirus vectors for diagnostic and therapeutic purposes, such as vaccines and expression systems.

BACKGROUND

Adenoviruses are double-stranded DNA viruses that have been isolated from a wide variety of avian and mammalian species, including swine. While the majority of adenovirus infections in swine are subclinical, porcine adenovirus (PAV) infection has been associated with encephalitis, pneumonia, kidney lesions and diarrhea. Derbyshire (1992) In: "Diseases of Swine" (ed. Leman *et al.*), 7th edition, Iowa State University Press, Ames, IA. pp. 225-227. Thus, there is a need for vaccines that will provide protection against PAV infection.

In addition to their potential ability to provide protection against PAV infection, PAVs could also be used as viral vaccine vectors, if insertion capacity can be determined, and appropriate insertion sites can be defined and characterized. It has been shown that PAV is capable of stimulating both humoral response and a mucosal antibody responses in the intestine of infected piglets. Tuboly *et al.* (1993) *Res. in Vet. Sci.* 54:345-350. Thus, recombinant PAV vaccine vectors would be especially useful, as they would be likely to be capable of providing both systemic and mucosal immunity to antigens encoded by native and/or recombinant PAV genomes.

Cross-neutralization studies have indicated the existence of at least five serotypes of PAV. Derbyshire *et al.* (1975) *J. Comp. Pathol.* 85:437-443; and

Hirahara *et al.* (1990) *Jpn. J. Vet. Sci.* **52**:407-409. Previous studies of the PAV genome have included the determination of restriction maps for PAV Type 3 (PAV-3) and cloning of restriction fragments representing the complete genome of PAV-3. Reddy *et al.* (1993) *Intervirology* **36**:161-168. In addition, restriction maps for PAV-1 and PAV-2 have been determined. Reddy *et al.* (1995*b*) *Arch. Virol.* **140**:195-200.

Nucleotide sequences have been determined for segments of the genome of various PAV serotypes. Sequences of the E3, pVIII and fiber genes of PAV-3 were determined by Reddy *et al.* (1995*a*) *Virus Res.* **36**:97-106. The E3, pVIII and fiber genes of PAV-1 and PAV-2 were sequenced by Reddy *et al.* (1996) *Virus Res.* **43**:99-109; while the PAV-4 E3, pVIII and fiber gene sequences were determined by Kleiboeker (1994) *Virus Res.* **31**:17-25. The PAV-4 fiber gene sequence was determined by Kleiboeker (1995*b*) *Virus Res.* **39**:299-309. Inverted terminal repeat (ITR) sequences for all five PAV serotypes (PAV-1 through PAV-5) were determined by Reddy *et al.* (1995*c*) *Virology* **212**:237-239. The PAV-3 penton sequence was determined by McCoy *et al.* (1996*a*) *Arch. Virol.* **141**:1367-1375. The nucleotide sequence of the E1 region of PAV-4 was determined by Kleiboeker (1995*a*) *Virus Res.* **36**:259-268. The sequence of the protease (23K) gene of PAV-3 was determined by McCoy *et al.* (1996*b*) *DNA Seq.* **6**:251-254. The unpublished sequence of the PAV-3 hexon gene (and the 14 N-terminal codons of the 23K protease gene) has been deposited in the GenBank database under accession No. U34592. The unpublished sequence of the PAV-3 100K gene has been deposited in the GenBank database under accession No. U82628. The sequence of the PAV-3 E4 region has been determined by Reddy *et al.* (1997) *Virus Genes* **15**:87-90.

Adenoviruses have proven to be effective vectors for the delivery and expression of foreign genes in a number of specific applications, and have a number of advantages as potential gene transfer and vaccine vectors. *See* Gerard *et al* (1993) *Trends Cardiovasc. Med.* **3**:171-177; Imler *et al.* (1995) *Hum. Gene Ther.* **6**:711-721. The ability of these vectors to mediate the efficient expression

of candidate therapeutic or vaccine genes in a variety of cell types, including post mitotic cells, is considered an advantage over other gene transfer vectors. Adenoviral vectors are divided into helper-independent and helper-dependent groups based on the region of the adenoviral genome used for the insertion of transgenes. Helper-dependent vectors are usually made by deletion of El sequences and substitution of foreign DNA, and are produced in complementing human cell lines that constitutively express El proteins. Graham *et al.* (1977) *J. Gen. Virol.* **36**:59-74; Fallaux *et al.* (1996) *Hum. Gene Ther.* **7**:215-222; Fallaux *et al.* (1998) *Hum. Gene Ther.* **9**:1909-1917. However, porcine adenoviruses do not replicate in human cell lines; hence these lines are unsuitable for the propagation of E1-deleted PAV vectors.

Though E1-deleted viruses do not replicate in cells that do not express El proteins, the viruses can express foreign proteins in these cells, provided the genes are placed under the control of a constitutive promoter. Xiang *et al.* (1996) *Virology* **219**:220-227. Vaccination of animals with adenovirus recombinants containing inserts in the E1 region induced a systemic immune response and provided protection against subsequent challenge. Imler *et al* (1995) *Hum.Gene Ther.* **6**:711-721; Imler *et al.* (1996) *Gene Therap* **3**:75-84.. This type of expression vector provides a significant safety profile to the vaccine as it eliminates the potential for dissemination of the vector within the vaccinee and therefore, the spread of the vector to nonvaccinated contacts or to the general environment. However, the currently used human adenovirus (HAV) based vectors are endemic in most populations, which provides an opportunity for recombination between the helper-dependent viral vectors and wild type viruses. To circumvent some of the problems associated with the use of human adenoviruses, non human adenoviruses have been explored as possible expression vectors. All vectors developed to date, except one (Klonjkowski *et al* (1997) *Hum. Gene Ther.* **8**:2103-2115), contain an intact El region. Use of such vectors for gene therapy in humans and vaccination in animals is unsafe because they

have the ability to replicate in normal cells, and they retain the oncogenic potential of the E1 region.

Recombinant PAV genomes containing heterologous nucleotide sequences have not yet been described. Similarly, sites where insertion of heterologous sequence would not interfere with the ability of a PAV vector to stimulate an immune response against a determinant encoded by an inserted sequence have not been identified. Consequently, the development of effective recombinant PAV vectors for use in immunization, expression systems and gene therapy, awaits resolution of these issues. Similarly, there is a need for improved adenoviral vectors lacking E1 replication and oncogenic functions, for expression of transgenes in mammalian cells.

SUMMARY OF THE INVENTION

The present invention provides the complete nucleotide sequence of the porcine adenovirus type 3 (PAV-3) genome. Nucleic acid sequences that are substantially homologous to those comprising a PAV genome are also encompassed by the invention. Substantially homologous sequences include those capable of duplex and/or triplex formation with a nucleic acid comprising all or part of a PAV genome (or with its complement). As is known to those of skill in the art, duplex formation is influenced by hybridization conditions, particularly hybridization stringency. Factors affecting hybridization stringency are well-known to those of skill in the art. *See*, for example, Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual; Hames *et al.* 1985) Nucleic Acid Hybridisation: A Practical Approach, IRL Press Ltd., Oxford Accordingly, it is within the skill of the art to identify a sequence that is substantially homologous to a sequence from a PAV genome.

In addition, novel porcine adenovirus (PAV) expression vector systems comprising PAV genome sequences are disclosed herein. The PAV-3 sequence includes regions into which heterologous sequences can be inserted including, but not limited to, the E1, E3 and E4 regions, and the region between E4 and the right

end of the genome. The invention also provides non-essential regions which can be deleted to increase the capacity of a PAV vector for inserted heterologous sequences. These include, but are not limited to, the E3 and E4 regions, and the region between E4 and the right end of the genome. Essential regions, such as E1, can also be deleted, if virus bearing such deletions are propagated in helper cell lines supplying the deleted essential function. Thus, PAV genome sequences can be replaced by one or more foreign genes to generate recombinant PAV vectors expressing heterologous antigenic polypeptides (or antigenic fragments thereof) for the purposes of producing live recombinant virus, subunit vaccines, nucleic acid immunization, or other types of therapy. Multiple heterologous sequences can be inserted into the same, or different, locations in the genome, limited only by the capacity of the virus to accept heterologous sequences. This capacity can be expanded by deletion of viral sequences.

In addition, the invention provides PAV transcriptional and translational regulatory sequences which can be used for expression of heterologous genes that have been inserted into the vectors of the invention. Furthermore, the novel sequences of the present invention can be used for diagnostic purposes, to determine the presence of PAV antigens and/or PAV nucleic acids in a subject or biological sample.

In additional embodiments, the invention provides compositions providing immunity to PAV infection, through expression of antigenic PAV polypeptides. The invention also provides vectors comprising PAV genome sequences, including sequences encoding various PAV genes as well as PAV regulatory sequences, which are useful for controlling the expression of heterologous genes inserted into PAV vectors.

The invention provides defective recombinant PAV vectors that are deleted in their E1 region, as well as helper cell lines providing E1 function, in which such defective vectors can be propagated. Because these defective vectors replicate inefficiently in cells other than the helper cells, they are less likely to stimulate an immune response in a mammalian host. This makes them

particularly suitable for use as vaccine vectors. In addition, since the amount of nucleic acid that can be packaged into an adenovirus virion is limited, deletion of the E1 region expands the capacity of these defective vectors, enabling them to accept larger inserts of heterologous sequence. Additional deletions in other regions of the genome can be used to expand the capacity of these defective vectors still further.

The invention further provides methods for obtaining recombinant PAV vectors. In a preferred embodiment, heterologous nucleotide sequences are introduced, through recombinant DNA techniques, into a bacterial plasmid comprising a defined portion of the PAV genome. The recombinant plasmid, containing heterologous sequences flanked by PAV sequences, is introduced into a host cell in combination with a full-length PAV genome or a plasmid containing a full-length or nearly full-length PAV genome. Within the host cell, recombination between the plasmid and the PAV genome generates a recombinant PAV genome. Alternatively, recombinant PAV genomes can be constructed *in vitro*, using standard techniques in molecular biology and biotechnology.

The invention also provides methods for preparing live recombinant virus and subunit vaccines for inducing protective immune responses to an infectious organism in a mammalian subject. Protective immune responses include humoral (antibody) responses, cell-mediated responses, mucosal responses, or any combination of these. The methods involve insertion, into the porcine adenovirus genome, of heterologous nucleotide sequences encoding one or more protective antigenic determinants of a pathogen. The heterologous sequences are inserted in such a way as to come under the regulatory control of a PAV promoter, or the heterologous sequences are inserted in operative linkage to a eukaryotic transcriptional regulatory sequence. Translation of transcribed heterologous sequences can be controlled by PAV translational regulatory elements, or the heterologous sequence can include non-PAV sequences which regulate its translation.

In another aspect, the invention includes the use of recombinant porcine adenoviruses and recombinant PAV vectors for the expression of a nucleotide or amino acid sequence of interest in a cell system, such as, for example, production of antigen to be used in the preparation of antibodies, or production of antisense RNA.

The invention also includes an expression system comprising a porcine adenovirus expression vector wherein heterologous nucleotide sequences are inserted. The inserted heterologous sequences can comprise one or more regulatory elements for transcription and/or translation, or can be inserted so as to come under the control of PAV regulatory elements. Inserted regulatory elements can be those that are normally associated with the heterologous sequence, or a heterologous sequence can be juxtaposed to and placed in operative linkage with a regulatory element with which it is not normally associated, using standard recombinant DNA techniques. Heterologous sequences can be inserted into a full-length PAV genome, or into a PAV genome which has been deleted in one or more regions. A deletion in the PAV genome can be made to provide a site for insertion of a heterologous sequence, or simply to increase the capacity of the PAV vector to accommodate heterologous sequences inserted at another location.

The invention also provides recombinant PAV polypeptides including, but not limited to, those encoded by the following genes: E1A, E1B, E4, pIX, DBP, pTP, pol, IVa2, 52K, IIIA, pIII, pVII, pV, pX, pVI, and 33K. Such recombinant PAV polypeptides are produced in any eukaryotic expression vector known in the art, into which is inserted a PAV nucleotide sequence according to the invention. Also provided are methods and compositions for recombinant production of heterologous polypeptides and RNAs in a PAV vector. Expression of heterologous polypeptides and RNAs in a PAV vector can be regulated by endogenous PAV regulatory sequences, or by non-PAV sequences. Non-PAV regulatory sequences can be those which normally regulate the heterologous sequence, or they can be sequences that are not normally associated with the heterologous sequence in a regulatory capacity.

Thus, in one embodiment, the invention includes an expression system in which one or more regions of the PAV genome are deleted and replaced with heterologous sequences. In another embodiment, the invention includes a PAV expression system in which heterologous sequences are introduced into the PAV genome without the removal of any PAV sequences. Intergenic regions of the PAV genome comprising regulatory sequences are useful in the practice of the invention for controlling the expression of homologous and heterologous sequences.

The invention also includes recombinant vector systems comprising two or more nucleic acid molecules. In one embodiment, the vector system comprises two plasmids, the first containing a full-length or nearly full-length PAV genome and the second containing a segment of the PAV genome, such as the left end (including the E1 region) or the right end (including the E3 and/or E4 regions). Introduction of heterologous nucleotide sequences into the second plasmid, followed by co-transfection of both plasmids into a suitable host cell, will allow homologous recombination between the two plasmids to generate a viral genome containing inserted heterologous sequences. In another embodiment, the vector system comprises a full-length or nearly full-length PAV genome and a plasmid containing a segment of the PAV genome. Insertion of heterologous sequences into the plasmid, followed by co-transfection and homologous recombination, will generate recombinant PAV genomes as above.

Additional aspects of the invention provide a recombinant PAV comprising a heterologous sequence wherein the heterologous sequence encodes an antigenic determinant of a disease-causing organism; and a recombinant PAV comprising a heterologous sequence wherein the heterologous sequence encodes a foreign gene or fragment thereof. In further embodiments, the invention provides pharmaceutical compositions comprising recombinant PAV for producing an immune response in a mammalian host, the recombinant PAV comprising a heterologous nucleotide sequence encoding a protective determinant of a pathogenic organism. The heterologous sequence is expressed in quantities

sufficient for induction of a protective immune response, either through operative linkage to one or more non-PAV regulatory sequences, or through control by endogenous PAV regulatory sequences. The protective immune response can be humoral, cell-mediated and/or mucosal.

The recombinant PAV vectors of the invention will also allow the expression of various therapeutic polypeptides in a wide range of mammalian hosts and are thus useful in the practices of nucleic acid immunization and gene therapy. Exemplary hosts include, but are not limited to, human, equine, bovine, porcine, ovine, caprine, avian, and murine. Those of skill in the art are aware of various therapeutic polypeptides which can be usefully expressed in mammalian hosts. Such therapeutic polypeptides include, but are not limited to, coagulation factors, growth hormones, cytokines, lymphokines, tumor-suppressing polypeptides, cell receptors, ligands for cell receptors, protease inhibitors, antibodies, toxins, immunotoxins, dystrophins, cystic fibrosis transmembrane conductance regulator (CFTR) and immunogenic polypeptides.

The invention also provides diagnostic methods and compositions for the detection of PAV nucleic acids and proteins in a cell or biological sample. The PAV nucleotide sequences disclosed herein can be used as hybridization probes to detect PAV nucleic acids. In addition, the PAV nucleotide sequences disclosed herein encode PAV polypeptides, which can be used for the production of antibodies reactive with various PAV antigens. Such antibodies can be used to detect PAV antigens by immunoassay. Alternatively, PAV polypeptides themselves can be used in competitive immunoassays to detect the presence of PAV antigens in a cell or biological sample. PAV polypeptides can be produced by the PAV vectors of the invention, or can be produced in any mammalian expression vector known in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the complete nucleotide sequence of the PAV-3 genome **(SEQ ID NO: 1)**.

Figure 2 shows the transcriptional map of the PAV-3 genome derived from alignment of the sequences of cDNA clones with the genomic sequence, and nuclease protection mapping of viral transcripts. The PAV-3 genome is represented by the thick horizontal line, with the numbers below the line representing PAV-3 map units (*i.e.*, percentage of genome length from the left end). Rightward-reading transcription units are depicted above the line and leftward-reading transcription units are shown below the line.

Figure 3 shows immunoprecipitation of E1A and E1B proteins from various cell lines.

In Figure **3A**, proteins in cell lysates were separated by gel electrophoresis, and analyzed by immunoblotting using the DP11 monoclonal antibody, which recognizes the human adenovirus E1A protein. Lane 1: 293 cells (human cells transformed by HAV-5, which express adenovirus E1A and E1B); Lane 2: Fetal porcine retinal cells; Lane 3: VIDO R1 cells; Lane 4: 293 cells.

In Figure **3B**, proteins in cell lysates were separated by gel electrophoresis, and analyzed by immunoblotting using the DP17 monoclonal antibody, which recognizes the human adenovirus E1B protein. Lane 1: human 293 cells; Lane 2: Fetal porcine retinal cells; Lane 3: VIDO R1 cells; Lane 4: 293 cells.

Figure 4 shows a map of the plasmid pPAV-101.

Figure 5 shows a map of the plasmid pPAV-102.

Figure 6 shows a map of the plasmid pPAV-300.

Figure 7 shows proteins labeled after infection of VIDO R1 cells with a recombinant PAV containing the PRV gp50 gene inserted in the E3 region. Labeled proteins were separated by gel electrophoresis; an autoradiogram of the gel is shown. Lane 1: Molecular weight markers of 30K, 46K, 69K and 96K, in order of increasing molecular weight. Lane 2: Mock-infected cells, 12 hours post-infection. Lane 3: PAV-3-infected cells, 12 hours post-infection. Lane 4: cells infected with a recombinant PAV containing the PRV gp50 gene, 12 hours post-infection. Lane 5: cells infected with a recombinant PAV containing the PRV

gp50 gene, 16 hours post-infection. Lane 6: cells infected with a recombinant PAV containing the PRV gp50 gene, 24 hours post-infection.

Figure 8 provides a schematic diagram of the construction of an E1- and E3-deleted PAV vector with a green fluorescent protein gene insertion.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides the complete nucleotide sequence and transcriptional map of the porcine adenovirus type 3 (PAV-3) genome. The sequence comprises a linear, double-stranded DNA molecule of about 34,094 base pairs, as shown in Figure 1 (**SEQ ID NO: 1**). Previously-determined partial sequences can be aligned with the complete genomic sequence as shown in Table 1.

Table 1. Alignment of published PAV-3 sequences

GenBank Accession No.	PAV Gene(s) included within sequence	Reference	Genome coordinates
L43077	ITR	Reddy et al., 1995*c*	1-144
U24432	penton	McCoy et al., 1996*a*	13556-15283
U34592	hexon; N-terminal 14 codons of 23K (protease) gene	unpublished	19036-21896
U33016	protease (23K)	McCoy et al., 1996*b*	21897-22676
U82628	100K	unpublished	24056-26572
U10433	E3, pVIII, fiber	Reddy et al., 1995*a*	27089-31148
L43363	E4	Reddy et al., 1997	31064-34094

Knowledge of the PAV genome sequence is useful for both therapeutic and diagnostic procedures. Regions suitable for insertion and regulated expression of heterologous sequences have been identified. These regions include, but are not limited to the E1, E3 and E4 regions, and the region between the E4 region and the right end of the genome. A heterologous nucleotide

sequence, with respect to the PAV vectors of the invention, is one which is not normally associated with PAV sequences as part of the PAV genome. Heterologous nucleotide sequences include synthetic sequences. Regions encoding immunogenic PAV polypeptides, for use in immunodiagnostic procedures, have also been identified and are disclosed herein. These include the regions encoding the following PAV proteins: E1A, E1B, E4, pIX, DBP, pTP, pol, IVa2, 52K, IIIA, pIII, pVII, pV, pX, pVI, 33K, pVIII, hexon and fiber (see Table 2). Regions essential for viral replication, such as E1 and E2A, can be deleted to provide attenuated strains for use as vaccines. Nonessential regions, such as parts of the E3 and E4 regions, can be deleted to provide insertion sites, or to provide additional capacity for insertion at a site other than the deleted region. Deletions of viral sequences can be obtained by any method known in the art, including but not limited to restriction enzyme digestion and ligation, oligonucleotide-mediated deletion mutagenesis, and the like.

The practice of the present invention employs, unless otherwise indicated, conventional microbiology, immunology, virology, molecular biology, and recombinant DNA techniques which are within the skill of the art. These techniques are fully explained in the literature. See, *e.g.*, Maniatis et al., *Molecular Cloning: A Laboratory Manual* (1982); *DNA Cloning: A Practical Approach*, vols. I & II (D. Glover, ed.); *Oligonucleotide Synthesis* (N. Gait, ed. (1984)); *Nucleic Acid Hybridization* (B. Hames & S. Higgins, eds. (1985)); *Transcription and Translation* (B. Hames & S. Higgins, eds. (1984)); *Animal Cell Culture* (R. Freshney, ed. (1986)); Perbal, *A Practical Guide to Molecular Cloning* (1984); Ausubel, *et al.*, *Current Protocols In Molecular Biology*, John Wiley & Sons (1987, 1988, 1989, 1990, 1991, 1992, 1993, 1994, 1995, 1996); and Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd Edition); vols. I, II & III (1989).

Nucleotide Sequence, Genome Organization, and Transcription Map of Porcine Adenovirus Type 3 (PAV-3).

The complete nucleotide sequence of PAV-3 genome is 34,094 base pairs (bp) in length and has a base composition of 31.3% G, 32.5% C, 18.3% A, and 17.9% T. Thus, the sequence of the PAV-3 genome has a G+C content of 63.8%, which is unusually high when compared with the G+C content of many other animal adenoviruses. The genome termini share inverted terminal repeats (ITR) of 144 bp. Reddy *et al.*, 1995*c*, *supra*. The organization of the genome as determined by analysis of open reading frames (ORFs), nuclease protection mapping, and sequencing of cDNA clones, is summarized in Table 2 and Figure 2.

One important feature of PAV-3 genome is the presence of a short virion associated (VA) RNA gene between the splice acceptor sites of the precursor terminal protein (pTP) and 52 kDa protein genes (Figure 2). Expression of VA genes increases the kinetics of viral replication; thereby providing the potential for higher yields of recombinant gene products using the PAV vectors of the invention. The locations of the signature sequences present upstream and downstream of VA RNA genes indicate the VA RNA gene of PAV-3 is about 126 nucleotides (nt) in length. This is somewhat shorter than most VA RNAs, whose lengths are 163 ± 14 nts, however shorter VA RNAs have also been reported in HAV-10 and CELO virus. Ma *et al.* (1996) *J. Virol.* **70**:5083-5099; and Chiocca *et al.* (1996) *J. Virol.* **70**:2939-2949. The VA RNA genes were not found in the genomes of BAV-3, CAV-1, and OAV. Reddy *et al.* (1998) *J. Virol.* **72**:1394-1402; Morrison *et al.* (1997) *J. Gen. Virol.* **78**:873-878; and Vrati *et al.* (1996) *Virology* **220**:186-199.

In PAV-3 the major late transcript initiates at 17.7 map units (m.u.: an adenovirus map unit is 1% of genome length, starting from the left end of the genome). There are six 3'-coterminal families of late mRNAs, denoted L1 to L6 (see Figure 2). All mRNAs produced from the major late promoter (MLP) contain a tripartite leader sequence (TPL). The first portion of the TPL lies next

to the MLP and is 61 nts long. The second portion lies within the gene coding for pol and is 68 nt in length. The third portion is 99 nts long and is located within the gene coding for pTP. Thus the TPL of PAV-3 is 228 nt long and is derived from three exons located at 17.7, 20.9, and 28.1 m.u.

The MLP and TPL sequences can be used for expression of a heterologous sequence in a recombinant PAV vector or in any other adenoviral expression system.

Table 2: Transcriptional and Translational Features of the PAV-3 Genome

Region	Gene	Transcription start site	ATG	Splice donor site	Splice acceptor site	Poly(A) signal	Poly(A) addition site
E1A	229R	heterogeneous	533			1286	1307
	214R		533	1043	1140	1286	1307
E1B	202R	1382	1461			4085	4110, 4112
	474R	1382	1829			4085	4110, 4112
pIX	Pix	3377	3394			4085	4110, 4112
E2A	DBP	17011c	24041c	26949c, 24714c	24793c, 24051c	22560c	22536c
E2B	pTP	17011c	13638c	24949c, 24714c	24793c, 13772c	4075c	4053c
	pol	17011c	13638c	24949c, 24714c	24793†c, 13772†c	4075c	4053c
IVa2	IVa2	5867c	5711c	5699c	5441c	4075c	4053c
E3		27473				28765	28793
E4		33730c				31189c	31170c
L1	52K	6064	10629	9684	10606	13601	13627
	IIIA	6064	11719	9684	11715	13601	13627
L2	pIII	6064	13662	9684	13662	15698*	15735
	pVII	6064	15170	9684	15139	15698*	15735
L3	pV	6064	15819	9684	15793	18992	19013
	pX	6064	17783	9684	17776	18992	19013
	pVI	6064	18076	9684	18063	18992	19013
L4	Hexon	6064	19097	9684	19096	22544	22567
	Protease	6064	21934	9684	21931†	22544	22567
L5	100k	6064	24056	9684	24056	28765	28793
	33K	6064	26181	9684	26130	28765	29793
	pVIII	6064	27089	9684	26792	28765	28793
L6	Fiber	6064	28939	9684	28910	31143	31164

Notes:

* TTGTTT is present as a polyadenylation signal instead of AATAAA

† The splice acceptor sites for the *pol* and protease genes were determined based on consensus splice acceptor sequences

"c" refers to sequences on the complementary (leftward-reading) strand of the PAV genome.

Construction of recombinant PAV vectors

In one embodiment of the invention, a recombinant PAV vector is constructed by *in vivo* recombination between a plasmid and a PAV genome. Generally, heterologous sequences are inserted into a plasmid vector containing a portion of the PAV genome, which may or may not possess one or more deletions of PAV sequences. The heterologous sequences are inserted into the PAV insert portion of the plasmid vector, such that the heterologous sequences are flanked by PAV sequences that are adjacent on the PAV genome. The PAV sequences serve as "guide sequences," to direct insertion of the heterologous sequences to a particular site in the PAV genome; the insertion site being defined by the genomic location of the guide sequences.

The vector is generally a bacterial plasmid, allowing multiple copies of the cloned sequence to be produced. In one embodiment, the plasmid is co-transfected, into an appropriate host cell, with a PAV genome comprising a full-length or nearly full-length PAV genomic sequence. The PAV genome can be isolated from PAV virions, or can comprise a PAV genome that has been inserted into a plasmid, using standard techniques of molecular biology and biotechnology. Construction of a plasmid containing a PAV genome is described in Example 2, *infra*. Nearly full-length PAV genomic sequences can be deleted in regions such as E1, E3, E4 and the region between E4 and the right end of the genome, but will retain sequences required for replication and packaging. PAV genomes can be deleted in essential regions if the essential function can be supplied by a helper cell line.

Insertion of the cloned heterologous sequences into a viral genome occurs by *in vivo* recombination between a plasmid vector (containing heterologous sequences flanked by PAV guide sequences) and a PAV genome following co-transfection into a suitable host cell. The PAV genome contains inverted terminal repeat (ITR) sequences required for initiation of viral DNA replication (Reddy *et al.* (1995c), *supra*), and sequences involved in packaging of replicated viral genomes. Adenovirus packaging signals generally lie between the left ITR and

the E1A promoter. Incorporation of the cloned heterologous sequences into the PAV genome thus places the heterologous sequences into a DNA molecule containing viral replication and packaging signals, allowing generation of multiple copies of a recombinant PAV genome that can be packaged into infectious viral particles. Alternatively, incorporation of the cloned heterologous sequences into a PAV genome places these sequences into a DNA molecule that can be replicated and packaged in an appropriate helper cell line. Multiple copies of a single sequence can be inserted to improve yield of the heterologous gene product, or multiple heterologous sequences can be inserted so that the recombinant virus is capable of expressing more than one heterologous gene product. The heterologous sequences can contain additions, deletions and/or substitutions to enhance the expression and/or immunological effect of the expressed gene product(s).

Attachment of guide sequences to a heterologous sequence can also be accomplished by ligation *in vitro*. In this case, a nucleic acid comprising a heterologous sequence flanked by PAV guide sequences can be co-introduced into a host cell along with a PAV genome, and recombination can occur to generate a recombinant PAV vector. Introduction of nucleic acids into cells can be achieved by any method known in the art, including, but not limited to, microinjection, transfection, electroporation, $CaPO_4$ precipitation, DEAE-dextran, liposomes, particle bombardment, *etc.*

In one embodiment of the invention, a recombinant PAV expression cassette can be obtained by cleaving a wild-type PAV genome with an appropriate restriction enzyme to produce a PAV restriction fragment representing, for example, the left end or the right end of the genome comprising E1 or E3 gene region sequences, respectively. The PAV restriction fragment can be inserted into a cloning vehicle, such as a plasmid, and thereafter at least one heterologous sequence (which may or may not encode a foreign protein) can be inserted into the E1 or E3 region with or without an operatively-linked eukaryotic transcriptional regulatory sequence. The recombinant expression cassette is

contacted with a PAV genome and, through homologous recombination or other conventional genetic engineering methods, the desired recombinant is obtained. In the case wherein the expression cassette comprises the E1 region or some other essential region, recombination between the expression cassette and a PAV genome can occur within an appropriate helper cell line such as, for example, an E1-transformed cell line. Restriction fragments of the PAV genome other than those comprising the E1 or E3 regions are also useful in the practice of the invention and can be inserted into a cloning vehicle such that heterologous sequences can be inserted into the PAV sequences. These DNA constructs can then undergo recombination *in vitro* or *in vivo*, with a PAV genome either before or after transformation or transfection of an appropriate host cell.

The invention also includes an expression system comprising a porcine adenovirus expression vector wherein a heterologous nucleotide sequence, e.g. DNA, replaces part or all of the E3 region, part or all of the E1 region, part or all of the E2 region, part or all of the E4 region, part or all of the late region and/or part or all of the regions occupied by the pIX, DBP, pTP, pol, IVa2, 52K, IIIA, pIII, pVII, pV, pX, pVI, and 33K genes. The expression system can be used wherein the foreign nucleotide sequences, *e.g.* DNA, are optionally in operative linkage with a eukaryotic transcriptional regulatory sequence. PAV expression vectors can also comprise inverted terminal repeat (ITR) sequences and packaging sequences.

The PAV E1A, E1B, pIX, DBP, pTP, pol, IVa2, 52K, IIIA, pIII, pVII, pV, pX, pVI, and 33K genes are essential for viral replication. Therefore, PAV vectors comprising deletions in any of these genes, or which lack functions encoded by any of these genes, are grown in an appropriate complementing cell line (*i.e.*, a helper cell line). Most, if not all, of the open reading frames in the E3 and E4 regions of PAV-3 are non-essential for viral replication and, therefore, deletions in these regions can be constructed for insertion or to increase vector capacity, without necessitating the use of a helper cell line for growth of the viral vector.

In another embodiment, the invention provides a method for constructing a full-length clone of a PAV genome by homologous recombination *in vivo*. In this embodiment, two or more plasmid clones, containing overlapping segments of the PAV genome and together covering the entire genome, are introduced into an appropriate bacterial host cell. Approximately 30 base pairs of overlap is required for homologous recombination in *E. coli*. Chartier *et al.* (1996) *J. Virol.* **70**:4805-4810. Through *in vivo* homologous recombination, the PAV genome segments are joined to form a full-length PAV genome. In a further embodiment, a recombinant plasmid containing left-end sequences and right-end sequences of the PAV genome, separated by a unique restriction site, is constructed. This plasmid is digested with the restriction enzyme recognizing the unique restriction site, to generate a unit-length linear plasmid, which is introduced into a cell together with a full-length PAV genome. Homologous recombination within the cell will result in production of a recombinant plasmid containing a full-length PAV genome. Recombinant plasmids will also generally contain sequences specifying replication in a host cell and one or more selective markers, such as, for example, antibiotic resistance.

Suitable host cells include any cell that will support recombination between a PAV genome and a plasmid containing PAV sequences, or between two or more plasmids, each containing PAV sequences. Recombination is generally performed in procaryotic cells, such as *E. coli*, while transfection of a plasmid containing a viral genome, to generate virus particles, is conducted in eukaryotic cells, preferably mammalian cells, most preferably porcine cell cultures. The growth of bacterial cell cultures, as well as culture and maintenance of eukaryotic cells and mammalian cell lines are procedures which are well-known to those of skill in the art.

In one embodiment of the invention, a defective recombinant PAV vector is used for expression of heterologous sequences. The defective vector will be deleted in all or part of the E1 region. Construction of a deletion in the E1 region of PAV is described in Example 3, *infra*. Heterologous sequences can be inserted

so as to replace the deleted E1 region, and/or can be inserted at other sites in the PAV genome, preferably E3, E4 and/or the region between E4 and the right end of the genome. Defective vectors with E1 deletions are grown in helper cell lines, which provide E1 function.

Accordingly, in one embodiment of the invention, a number of recombinant helper cell lines are produced according to the present invention by constructing an expression cassette comprising an adenoviral E1 region and transforming host cells therewith to provide complementing cell lines or cultures providing E1 functions. The terms "complementing cell," "complementing cell line," "helper cell" and "helper cell line" are used interchangeably herein to denote a cell line that provides a viral function that is deficient in a deleted PAV, preferably E1 function. These recombinant complementing cell lines are capable of allowing a defective recombinant PAV, having a deleted E1 gene region, wherein the deleted sequences are optionally replaced by heterologous nucleotide sequences, to replicate and express one or more foreign genes or fragments thereof encoded by the heterologous nucleotide sequences. PAV vectors with E1 deletions, wherein heterologous sequences are inserted in regions other than E1, can also be propagated in these complementing cell lines, and will express the heterologous sequences if they are inserted downstream of a PAV promoter or are inserted in operative linkage with a eukaryotic regulatory sequence. Preferred helper cell lines include VIDO R1 cells, as described in Example 1, *infra*. Briefly, the VIDO R1 cell line is a porcine retinal cell line that has been transfected with DNA from the human adenovirus type 5 (HAV-5) E1 region, and which supports the growth of PAV E1A deletions and HAV-5 E1 deletions.

Transformation of porcine cells with either PAV or HAV has not been reported due to the fact that exposure of permissive or semi-permissive cells to adenovirus normally leads to lysis of infected cells. Graham *et al., supra*. The approach used in the present study to create a PAV E1-complementing cell line employing the E1 region of HAV-5 is novel as E1A proteins of HAV-5 have been shown for the first time to complement PAV-3 E1 mutants. There are several

reasons that the E1 region of HAV-5 was used for transformation of porcine embryonic retinal cells. The E1 region of HAV-5 was shown to transform human retina cells very efficiently. Fallaux *et al.* (1998) *supra.* In contrast to the E1 region of PAV-3, the E1 region of HAV-5 has been thoroughly characterized and the monoclonal antibodies against the E1 proteins are readily available from commercial sources. In addition, the E1A region of HAV-5 was shown to complement the E1A functions of several non-human adenoviruses. Ball *et al.* (1988) *J. Virol.* **62**:3947-3957; Zheng *et al.* (1994) *Virus Res.* **31**:163-186.

More generally, defective recombinant PAV vectors, lacking one or more essential functions encoded by the PAV genome, can be propagated in appropriate complementing cell lines, wherein a particular complementing cell line provides a function or functions that is (are) lacking in a particular defective recombinant PAV vector. Complementing cell lines can provide viral functions through, for example, co-infection with a helper virus, or by integrating or otherwise maintaining in stable form a fragment of a viral genome encoding a particular viral function.

In another embodiment of the invention, E1 function (or the function of any other viral region which may be mutated or deleted in any particular viral vector) can be supplied (to provide a complementing cell line) by co-infection of cells with a virus which expresses the function that the vector lacks.

PAV expression systems

In one embodiment, the present invention identifies and provides means of deleting regions of the PAV genome, to provide sites into which heterologous or homologous nucleotide sequences encoding foreign genes or fragments thereof can be inserted to generate porcine adenovirus recombinants. In preferred embodiments, deletions are made in part or all of the nucleotide sequences of the PAV E1, E3, or E4 regions and/or the region between E4 and the right end of genome. E1 deletion is described in Example 3; E3 deletion and insertion of heterologous sequence in the E3 region are described in Example 4 and 5; and

insertion of a heterologous sequence between the E4 region and the right end of the PAV genome, as well as expression of the inserted sequence, is described in Example 6, *infra*.

In another embodiment, the invention identifies and provides additional regions of the PAV genome (and fragments thereof) suitable for insertion of heterologous or homologous nucleotide sequences encoding foreign genes or fragments thereof to generate PAV recombinants. These regions include nucleotides 145-13,555; 15,284-19,035; 22,677-24,055; 26,573-27,088; and 31,149-34,094 and comprise the E2 region, the late region, and genes encoding the pIX, DBP, pTP, pol, IVa2, 52K, IIIA, pIII, pVII, pV, pX, pVI, and 33K proteins. These regions of the PAV genome can be used, among other things, for insertion of foreign sequences, for provision of DNA control sequences including transcriptional and translational regulatory sequences, or for diagnostic purposes to detect the presence, in a biological sample, of viral nucleic acids and/or proteins encoded by these regions. Example 7, *infra*, describes procedures for constructing insertions in these regions.

One or more heterologous sequences can be inserted into one or more regions of the PAV genome to generate a recombinant PAV vector, limited only by the insertion capacity of the PAV genome and ability of the recombinant PAV vector to express the inserted heterologous sequences. In general, adenovirus genomes can accept inserts of approximately 5% of genome length and remain capable of being packaged into virus particles. The insertion capacity can be increased by deletion of non-essential regions and/or deletion of essential regions whose function is provided by a helper cell line.

In one embodiment of the invention, insertion can be achieved by constructing a plasmid containing the region of the PAV genome into which insertion is desired. The plasmid is then digested with a restriction enzyme having a recognition sequence in the PAV portion of the plasmid, and a heterologous sequence is inserted at the site of restriction digestion. The plasmid, containing a portion of the PAV genome with an inserted heterologous sequence,

in co-transformed, along with a plasmid (such as pPAV-200) containing a full-length PAV genome, into a bacterial cell (such as, for example, *E. coli*), wherein homologous recombination between the plasmids generates a full-length PAV genome containing inserted heterologous sequences.

Deletion of PAV sequences, to provide a site for insertion of heterologous sequences or to provide additional capacity for insertion at a different site, can be accomplished by methods well-known to those of skill in the art. For example, for PAV sequences cloned in a plasmid, digestion with one or more restriction enzymes (with at least one recognition sequence in the PAV insert) followed by ligation will, in some cases, result in deletion of sequences between the restriction enzyme recognition sites. Alternatively, digestion at a single restriction enzyme recognition site within the PAV insert, followed by exonuclease treatment, followed by ligation will result in deletion of PAV sequences adjacent to the restriction site. A plasmid containing one or more portions of the PAV genome with one or more deletions, constructed as described above, can be co-transfected into a bacterial cell along with a plasmid containing a full-length PAV genome to generate, by homologous recombination, a plasmid containing a PAV genome with a deletion at a specific site. PAV virions containing the deletion can then be obtained by transfection of mammalian cells (such as ST or VIDO R1 cells) with the plasmid containing a PAV genome with a deletion at a specific site.

Expression of an inserted sequence in a recombinant PAV vector will depend on the insertion site. Accordingly, preferred insertion sites are adjacent to and downstream (in the transcriptional sense) of PAV promoters. The transcriptional map of PAV, as disclosed herein, provides the locations of PAV promoters. Locations of restriction enzyme recognition sequences downstream of PAV promoters, for use as insertion sites, can be easily determined by one of skill in the art from the PAV nucleotide sequence provided herein. Alternatively, various *in vitro* techniques can be used for insertion of a restriction enzyme recognition sequence at a particular site, or for insertion of heterologous sequences at a site that does not contain a restriction enzyme recognition

sequence. Such methods include, but are not limited to, oligonucleotide-mediated heteroduplex formation for insertion of one or more restriction enzyme recognition sequences (*see*, for example, Zoller *et al.* (1982) *Nucleic Acids Res.* **10**:6487-6500; Brennan *et al.* (1990) *Roux's Arch. Dev. Biol.* **199**:89-96; and Kunkel *et al.* (1987) *Meth. Enzymology* **154**:367-382) and PCR-mediated methods for insertion of longer sequences. *See*, for example, Zheng *et al.* (1994) *Virus Research* **31**:163-186.

It is also possible to obtain expression of a heterologous sequence inserted at a site that is not downstream from a PAV promoter, if the heterologous sequence additionally comprises transcriptional regulatory sequences that are active in eukaryotic cells. Such transcriptional regulatory sequences can include cellular promoters such as, for example, the bovine hsp70 promoter and viral promoters such as, for example, herpesvirus, adenovirus and papovavirus promoters and DNA copies of retroviral long terminal repeat (LTR) sequences.

In another embodiment, homologous recombination in a procaryotic cell can be used to generate a cloned PAV genome; and the cloned PAV-3 genome can be propagated as a plasmid. Infectious virus can be obtained by transfection of mammalian cells with the cloned PAV genome rescued from plasmid-containing cells. Example 2, *infra* describes construction of an infectious plasmid containing a PAV-3 genome.

The invention provides PAV regulatory sequences which can be used to regulate the expression of heterologous genes. A regulatory sequence can be, for example, a transcriptional regulatory sequence, a promoter, an enhancer, an upstream regulatory domain, a splicing signal, a polyadenylation signal, a transcriptional termination sequence, a translational regulatory sequence, a ribosome binding site and a translational termination sequence.

Therapeutic genes and polypeptides

The PAV vectors of the invention can be used for the expression of therapeutic polypeptides in applications such as *in vitro* polypeptide production,

vaccine production, nucleic acid immunization and gene therapy, for example. Therapeutic polypeptides comprise any polypeptide sequence with therapeutic and/or diagnostic value and include, but are not limited to, coagulation factors, growth hormones, cytokines, lymphokines, tumor-suppressing polypeptides, cell receptors, ligands for cell receptors, protease inhibitors, antibodies, toxins, immunotoxins, dystrophins, cystic fibrosis transmembrane conductance regulator (CFTR) and immunogenic polypeptides.

In a preferred embodiment, PAV vectors will contain heterologous sequences encoding protective determinants of various pathogens of swine, for use in subunit vaccines and nucleic acid immunization. Representative swine pathogen antigens include, but are not limited to, pseudorabies virus (PRV) gp50; transmissible gastroenteritis virus (TGEV) S gene; porcine rotavirus VP7 and VP8 genes; genes of porcine respiratory and reproductive syndrome virus (PRRS), in particular ORF 5; genes of porcine epidemic diarrhea virus; genes of hog cholera virus, and genes of porcine parvovirus.

Various foreign genes or nucleotide sequences or coding sequences (prokaryotic, and eukaryotic) can be inserted into a PAV vector, in accordance with the present invention, particularly to provide protection against a wide range of diseases. Many such genes are already known in the art; the problem heretofore having been to provide a safe, convenient and effective vaccine vector for the genes or sequences.

A heterologous (*i.e.*, foreign) nucleotide sequence can consist of one or more gene(s) of interest, and preferably of therapeutic interest. In the context of the present invention, a gene of interest can code either for an antisense RNA, a ribozyme or for an mRNA which will then be translated into a protein of interest. A gene of interest can be of genomic type, of complementary DNA (cDNA) type or of mixed type (minigene, in which at least one intron is deleted). It can code for a mature protein, a precursor of a mature protein, in particular a precursor intended to be secreted and accordingly comprising a signal peptide, a chimeric protein originating from the fusion of sequences of diverse origins, or a mutant of

a natural protein displaying improved or modified biological properties. Such a mutant can be obtained by deletion, substitution and/or addition of one or more nucleotide(s) of the gene coding for the natural protein, or any other type of change in the sequence encoding the natural protein, such as, for example, transposition or inversion.

A gene of interest can be placed under the control of regulatory sequences suitable for its expression in a host cell. Suitable regulatory sequences are understood to mean the set of elements needed for transcription of a gene into RNA (ribozyme, antisense RNA or mRNA), for processing of RNA, and for the translation of an mRNA into protein. Among the elements needed for transcription, the promoter assumes special importance. It can be a constitutive promoter or a regulatable promoter, and can be isolated from any gene of eukaryotic, prokaryotic or viral origin, and even adenoviral origin. Alternatively, it can be the natural promoter of the gene of interest. Generally speaking, a promoter used in the present invention can be chosen to contain cell-specific regulatory sequences, or modified to contain such sequences. For example, a gene of interest for use in the present invention is placed under the control of an immunoglobulin gene promoter when it is desired to target its expression to lymphocytic host cells. There may also be mentioned the HSV-1 TK (herpesvirus type 1 thymidine kinase) gene promoter, the adenoviral MLP (major late promoter), in particular of human adenovirus type 2, the RSV (Rous Sarcoma Virus) LTR (long terminal repeat), the CMV (Cytomegalovirus) early promoter, and the PGK (phosphoglycerate kinase) gene promoter, for example, permitting expression in a large number of cell types.

Alternatively, targeting of a recombinant PAV vector to a particular cell type can be achieved by constructing recombinant hexon and/or fiber genes. The protein products of these genes are involved in host cell recognition; therefore, the genes can be modified to contain peptide sequences that will allow the virus to recognize alternative host cells.

Among genes of interest which are useful in the context of the present invention, there may be mentioned:

- genes coding for cytokines such as interferons and interleukins;
- genes encoding lymphokines;
- genes coding for membrane receptors such as the receptors recognized by pathogenic organisms (viruses, bacteria or parasites), preferably by the HIV virus (human immunodeficiency virus);
- genes coding for coagulation factors such as factor VIII and factor IX;
- genes coding for dystrophins;
- genes coding for insulin;
- genes coding for proteins participating directly or indirectly in cellular ion channels, such as the CFTR (cystic fibrosis transmembrane conductance regulator) protein;
- genes coding for antisense RNAs, or proteins capable of inhibiting the activity of a protein produced by a pathogenic gene which is present in the genome of a pathogenic organism, or proteins (or genes encoding them) capable of inhibiting the activity of a cellular gene whose expression is deregulated, for example an oncogene;
- genes coding for a protein inhibiting an enzyme activity, such as α_1-antitrypsin or a viral protease inhibitor, for example;
- genes coding for variants of pathogenic proteins which have been mutated so as to impair their biological function, such as, for example, trans-dominant variants of the *tat* protein of the HIV virus which are capable of competing with the natural protein for binding to the target sequence, thereby preventing the activation of HIV;
- genes coding for antigenic epitopes in order to increase the host cell's immunity;
- genes coding for major histocompatibility complex classes I and II proteins, as well as the genes coding for the proteins which are inducers of these genes;

- genes coding for antibodies;
- genes coding for immunotoxins;
- genes encoding toxins;
- genes encoding growth factors or growth hormones;
- genes encoding cell receptors and their ligands;
- genes encoding tumor suppressors;
- genes coding for cellular enzymes or those produced by pathogenic organisms; and
- suicide genes. The HSV-1 TK suicide gene may be mentioned as an example. This viral TK enzyme displays markedly greater affinity compared to the cellular TK enzyme for certain nucleoside analogues (such as acyclovir or gancyclovir). It converts them to monophosphorylated molecules, which can themselves be converted by cellular enzymes to nucleotide precursors, which are toxic. These nucleotide analogues can be incorporated into replicating DNA molecules, hence incorporation occurs chiefly in the DNA of dividing cells. This incorporation can result in specific destruction of dividing cells such as cancer cells.

This list is not restrictive, and any other gene of interest can be used in the context of the present invention. In some cases the gene for a particular antigen can contain a large number of introns or can be from an RNA virus, in these cases a complementary DNA copy (cDNA) can be used. It is also possible that only fragments of nucleotide sequences of genes can be used (where these are sufficient to generate a protective immune response or a specific biological effect) rather than the complete sequence as found in the wild-type organism. Where available, synthetic genes or fragments thereof can also be used. However, the present invention can be used with a wide variety of genes, fragments and the like, and is not limited to those set out above.

Recombinant PAV vectors can be used to express antigens for provision of, for example, subunit vaccines. Antigens used in the present invention can be either native or recombinant antigenic polypeptides or fragments. They can be

partial sequences, full-length sequences, or even fusions (e.g., having appropriate leader sequences for the recombinant host, or with an additional antigen sequence for another pathogen). The preferred antigenic polypeptide to be expressed by the virus systems of the present invention contain full-length (or near full-length) sequences encoding antigens. Alternatively, shorter sequences that are antigenic (i.e., encode one or more epitopes) can be used. The shorter sequence can encode a "neutralizing epitope," which is defined as an epitope capable of eliciting antibodies that neutralize virus infectivity in an *in vitro* assay. Preferably the peptide should encode a "protective epitope" that is capable of raising in the host a "protective immune response;" i.e., a humoral (*i.e.* antibody-mediated), cell-mediated, and/or mucosal immune response that protects an immunized host from infection.

The antigens used in the present invention, particularly when comprised of short oligopeptides, can be conjugated to a vaccine carrier. Vaccine carriers are well known in the art: for example, bovine serum albumin (BSA), human serum albumin (HSA) and keyhole limpet hemocyanin (KLH). A preferred carrier protein, rotavirus VP6, is disclosed in EPO Pub. No. 0259149, the disclosure of which is incorporated by reference herein.

Genes for desired antigens or coding sequences thereof which can be inserted include those of organisms which cause disease in mammals, particularly porcine pathogens such as pseudorabies virus (PRV), transmissible gastroenteritis virus (TGEV), porcine rotavirus, porcine respiratory and reproductive syndrome virus (PRRS), porcine epidemic diarrhea virus (PEDV), hog cholera virus (HCV), porcine parvovirus and the like. Genes encoding antigens of human pathogens are also useful in the practice of the invention.

Therapeutic applications

With the recombinant viruses of the present invention, it is possible to provide protection against a wide variety of diseases affecting swine, cattle, humans and other mammals. Any of the recombinant antigenic determinants or

recombinant live viruses of the invention can be formulated and used in substantially the same manner as described for the antigenic determinant vaccines or live vaccine vectors.

The present invention also includes pharmaceutical compositions comprising a therapeutically effective amount of a recombinant vector, recombinant virus or recombinant protein, prepared according to the methods of the invention, in combination with a pharmaceutically acceptable vehicle and/or an adjuvant. Such a pharmaceutical composition can be prepared and dosages determined according to techniques that are well-known in the art. The pharmaceutical compositions of the invention can be administered by any known administration route including, but not limited to, systemically (for example, intravenously, intratracheally, intraperitoneally, intranasally, parenterally, enterically, intramuscularly, subcutaneously, intratumorally or intracranially) or by aerosolization or intrapulmonary instillation. Administration can take place in a single dose or in doses repeated one or more times after certain time intervals. The appropriate administration route and dosage will vary in accordance with the situation (for example, the individual being treated, the disorder to be treated or the gene or polypeptide of interest), but can be determined by one of skill in the art.

The vaccines of the invention carrying foreign genes or fragments can be orally administered in a suitable oral carrier, such as in an enteric-coated dosage form. Oral formulations include such normally-employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin cellulose, magnesium carbonate, and the like. Oral vaccine compositions may be taken in the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders, containing from about 10% to about 95% of the active ingredient, preferably about 25% to about 70%. An oral vaccine may be preferable to raise mucosal immunity (which plays an important role in protection against pathogens infecting the gastrointestinal tract) in combination with systemic immunity.

In addition, the vaccine can be formulated into a suppository. For suppositories, the vaccine composition will include traditional binders and carriers, such as polyalkaline glycols or triglycerides. Such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10% (w/w), preferably about 1% to about 2%.

Protocols for administering to animals the vaccine composition(s) of the present invention are within the skill of the art in view of the present disclosure. Those skilled in the art will select a concentration of the vaccine composition in a dose effective to elicit antibody, cell-mediated and/or mucosal immune responses to the antigenic fragment. Within wide limits, the dosage is not believed to be critical. Typically, the vaccine composition is administered in a manner which will deliver between about 1 to about 1,000 micrograms of the subunit antigen in a convenient volume of vehicle, *e.g.*, about 1-10 ml. Preferably, the dosage in a single immunization will deliver from about 1 to about 500 micrograms of subunit antigen, more preferably about 5-10 to about 100-200 micrograms (e.g., 5-200 micrograms).

The timing of administration may also be important. For example, a primary inoculation preferably may be followed by subsequent booster inoculations, for example, several weeks to several months after the initial immunization, if needed. To insure sustained high levels of protection against disease, it may be helpful to readminister booster immunizations at regular intervals, for example once every several years. Alternatively, an initial dose may be administered orally followed by later inoculations, or vice versa. Preferred vaccination protocols can be established through routine vaccination protocol experiments.

The dosage for all routes of administration of *in vivo* recombinant virus vaccine depends on various factors including, the size of patient, nature of infection against which protection is needed, carrier and the like and can readily be determined by those of skill in the art. By way of non-limiting example, a dosage of between approximately 10^3 pfu and 10^8 pfu can be used. As with *in*

vitro subunit vaccines, additional dosages can be given as determined by the clinical factors involved.

A problem that has beset the use of adenovirus vectors for immunization and gene therapy in humans is the rapid development of an immunological response (or indeed in some cases existing immunity) to human adenoviruses (HAVs). Recombinant PAV vectors are likely to be less immunogenic in humans and, for this and other reasons, will be useful either as a substitute for HAV vectors or in combination with HAV vectors. For example, an initial immunization with a HAV vector can be followed by booster immunizations using PAV vectors; alternatively, initial immunization with a recombinant PAV vector can be followed by booster immunizations with HAV and/or PAV vectors.

The presence of low levels of helper-independent vectors in the batches of helper-dependent human adenoviruses that are grown in complementing human cell lines has been reported. Fallaux et *al.* (1998) *supra.* This occurs as a result of recombination events between the viral DNA and the integrated adenoviral sequences present in the complementing cell line. Hehir *et al.* (1996) *J. Virol.* **70**:8459-8467. This type of contamination constitutes a safety risk, which could result in the replication and spread of the virus. Complete elimination of helper-dependent adenoviruses in the batches of helper-dependent vectors can be achieved using two approaches. The first is by developing new helper cell lines and matched vectors that do not share any common sequences. Fallaux *et al.* (1998) *supra.* The second approach is to take advantage of possible cross-complementation between two distantly related adenoviruses such as HAV-5 and PAV-3. VIDO R1 cells contain the E1 coding sequences of HAV-5. Although there is no significant homology between the E1 regions of HAV-5 and PAV-3 at the nucleotide sequence level, the proteins produced from the region can complement each others' function(s). Thus, the problem of helper-independent vector generation by homologous recombination is eliminated when VIDO R1 cells are used for the propagation of recombinant PAV-3.

The invention also encompasses a method of treatment, according to which a therapeutically effective amount of a PAV vector, recombinant PAV, or host cell of the invention is administered to a mammalian subject requiring treatment. The finding that PAV-3 was effective in entering canine, sheep and bovine cells in which it does not replicate or replicates poorly is an important observation. *See* Example 8, *infra.* This may have implications in designing PAV-3 vectors for vaccination in these and other animal species.

PAV Expression Systems

Recombinant PAV vectors can be used for regulated expression of foreign polypeptides encoded by heterologous nucleotide sequences. Standard conditions of cell culture, such as are known to those of skill in the art, will allow maximal expression of recombinant polypeptides . They can be used, in addition, for regulated expression of RNAs encoded by heterologous nucleotide sequences, as in, for example, antisense applications and expression of ribozymes.

When the heterologous sequences encode an antigenic polypeptide, PAV vectors comprising insertions of heterologous nucleotide sequences can be used to provide large quantities of antigen which are useful, in turn, for the preparation of antibodies. Methods for preparation of antibodies are well-known to those of skill in the art. Briefly, an animal (such as a rabbit) is given an initial subcutaneous injection of antigen plus Freund's complete adjuvant. One to two subsequent injections of antigen plus Freund's incomplete adjuvant are given at approximately 3 week intervals. Approximately 10 days after the final injection, serum is collected and tested for the presence of specific antibody by ELISA, Western Blot, immunoprecipitation, or any other immunological assay known to one of skill in the art.

Adenovirus E1 gene products transactivate many cellular genes; therefore, cell lines which constitutively express E1 proteins can express cellular polypeptides at a higher levels than other cell lines. The recombinant mammalian, particularly porcine, cell lines of the invention can be used to prepare and isolate

polypeptides, including those such as (a) proteins associated with adenovirus E1A proteins: *e.g.* p300, retinoblastoma (Rb) protein, cyclins, kinases and the like; (b) proteins associated with adenovirus E1B protein: *e.g.* p53 and the like; growth factors, such as epidermal growth factor (EGF), transforming growth factor (TGF) and the like; (d) receptors such as epidermal growth factor receptor (EGF-R), fibroblast growth factor receptor (FGF-R), tumor necrosis factor receptor (TNF-R), insulin-like growth factor receptor (IGF-R), major histocompatibility complex class I receptor and the like; (e) proteins encoded by proto-oncogenes such as protein kinases (tyrosine-specific protein kinases and protein kinases specific for serine or threonine), p21 proteins (guanine nucleotide-binding proteins with GTPase activity) and the like; (f) other cellular proteins such as actins, collagens, fibronectins, integrins, phosphoproteins, proteoglycans, histones and the like, and (g) proteins involved in regulation of transcription such as TATA-box-binding protein (TBP), TBP-associated factors (TAFs), Sp1 binding protein and the like.

Gene Therapy

The invention also includes a method for providing gene therapy to a mammal, such as a porcine, human or other mammal in need thereof, to control a gene deficiency. In one embodiment, the method comprises administering to said mammal a live recombinant porcine adenovirus containing a heterologous nucleotide sequence encoding a non-defective form of said gene under conditions wherein the recombinant virus vector genome is incorporated into said mammalian genome or is maintained independently and extrachromosomally to provide expression of the required gene in the target organ or tissue. These kinds of techniques are currently being used by those of skill in the art to replace a defective gene or portion thereof. Examples of foreign genes, heterologous nucleotide sequences, or portions thereof that can be incorporated for use in gene therapy include, but are not limited to, cystic fibrosis transmembrane conductance regulator gene, human minidystrophin gene, alpha-1-antitrypsin gene and the like.

In particular, the practice of the present invention in regard to gene therapy in humans is intended for the prevention or treatment of diseases including, but not limited to, genetic diseases (for example, hemophilia, thalassemias, emphysema, Gaucher's disease, cystic fibrosis, Duchenne muscular dystrophy, Duchenne's or Becker's myopathy, *etc.*), cancers, viral diseases (for example, AIDS, herpesvirus infection, cytomegalovirus infection and papillomavirus infection) and the like. For the purposes of the present invention, the vectors, cells and viral particles prepared by the methods of the invention may be introduced into a subject either *ex vivo*, (*i.e.*, in a cell or cells removed from the patient) or directly *in vivo* into the body to be treated. Preferably, the host cell is a human cell and, more preferably, is a lung, fibroblast, muscle, liver or lymphocytic cell or a cell of the hematopoietic lineage.

Diagnostic applications

The PAV genome, or any subregion of the PAV genome, is suitable for use as a nucleic acid probe, to test for the presence of PAV nucleic acid in a subject or a biological sample. The presence of viral nucleic acids can be detected by techniques known to one of skill in the art including, but not limited to, hybridization assays, polymerase chain reaction, and other types of amplification reactions. Suitable labels and hybridization techniques are well-known to those of skill in the art. See, for example, Kessler (ed.), *Nonradioactive Labeling and Detection of Biomolecules,* Springer-Verlag, Berlin, 1992; Kricka (ed.) *Nonisotopic DNA Probe Techniques*, Academic Press, San Diego, 1992; Howard (ed.) *Methods in Nonradioactive Detection*, Appleton & Lange, Norwalk, 1993; Ausubel *et al., supra*; and Sambrook *et al., supra.* Diagnostic kits comprising the nucleotide sequences of the invention can also contain reagents for cell disruption and nucleic acid purification, as well as buffers and solvents for the formation, selection and detection of hybrids.

Regions of the PAV genome can be inserted into any expression vector known in the art and expressed to provide, for example, vaccine formulations,

protein for immunization, *etc.* The amino acid sequence of any PAV protein can be determined by one of skill in the art from the nucleotide sequences disclosed herein. PAV proteins can be used for diagnostic purposes, for example, to detect the presence of PAV antigens. Methods for detection of proteins are well-known to those of skill in the art and include, but are not limited to, various types of direct and competitive immunoassays, ELISA, Western blotting, enzymatic assay, immunohistochemistry, *etc.* *See*, for example, Harlow & Lane (eds.): Antibodies, A Laboratory Manual, Cold Spring Harbor Press, New York, 1988. Diagnostic kits comprising PAV polypeptides or amino acid sequences can also comprise reagents for protein isolation and for the formation, isolation, purification and/or detection of immune complexes.

EXAMPLES

METHODS

Virus and viral DNA.

The 6618 strain of PAV-3 was propagated in the swine testis (ST) cell line and in E1-transformed porcine retinal cells (VIDO R1, see below). Porcine embryonic retinal cells were obtained from the eyeballs of piglets delivered by caesarian section two weeks before the parturition date. Uninfected cells were grown in MEM supplemented with 10% fetal bovine serum (FBS). MEM with 2% FBS was used for maintenance of infected cells. Viral DNA was extracted either from infected cell monolayers by the method of Hirt (1967) *J. Mol. Biol.* **26**:365-369, or from purified virions as described by Graham *et al.* (1991) in "Methods in Molecular Biology" Vol. 7, Gene transfer and expression protocols, ed. E.J. Murray, Humana Press, Clifton, NJ, pp. 109-128.

Plasmids and genomic DNA sequencing.

Selected restriction enzyme fragments of PAV-3 DNA were cloned into pGEM-3Z and pGEM-7Zf(+) plasmids (Promega). Nucleotide sequences were determined on both strands of the genome by the dideoxy chain-termination

method using Sequenase® enzyme (U.S. Biochemicals) and the dye-terminator method with an Applied Biosystems (Foster City, CA) DNA sequencer.

cDNA library.

A cDNA library was generated from polyadenylated RNA extracted from PAV-3 infected ST cells at 12 h and 24 h post infection. Double stranded cDNAs were made with reagents from Stratagene and cloned into Lambda ZAP vector. Plaques which hybridized to specific restriction enzyme fragments of PAV-3 DNA were plaque purified twice. Plasmids containing cDNAs were excised from the Lambda ZAP vector according to the manufacturer's protocol. The resulting plasmid clones were characterized by restriction endonuclease analysis and by sequencing of both ends of the cDNA insert with T3- and T7-specific primers. Selected clones were sequenced with internal primers. cDNA sequences were aligned with genomic sequences to determine the transcription map.

Viral transcript mapping by nuclease protection

Transcript mapping was conducted according to the method of Berk *et al.* (1977) *Cell* 12:721-732.

Example 1: Development of an E1-complementing helper cell line (VIDO R1)

Primary cultures of porcine embryonic retina cells were transfected with 10 µg of plasmid pTG 4671 (Transgene, Strasbourg, France) by the calcium phosphate technique. The pTG 4671 plasmid contains the entire E1A and E1B sequences (nts 505-4034) of HAV-5, along with the puromycin acetyltransferase gene as a selectable marker. In this plasmid, the El region is under the control of the constitutive promoter from the mouse phosphoglycerate kinase gene, and the puromycin acetyltransferase gene is controlled by the constitutive SV40 early promoter. Transformed cells were selected by three passages in medium containing 7 µg/ml puromycin, identified based on change in their morphology

from single foci (*i.e.*, loss of contact inhibition), and subjected to single cell cloning. The established cell line was first tested for its ability to support the growth of El deletion mutants of HAV-5. Subsequently the cell line was further investigated for the presence of E1 sequences in the genome by PCR, expression of the E1A and E1B proteins by Western blot, and doubling time under cell culture conditions. E1 sequences were detected, and production of E1A and E1B proteins was demonstrated by immunoprecipitation (Figure 3). Doubling time was shorter, when compared to that of the parent cell line. Example 3, *infra*, shows that this cell line is capable of complementing a PAV E1A deletion mutant.

To assess the stability of El expression, VIDO R1 cells were cultured through more than 50 passages (split 1:3 twice weekly) and tested for their ability to support the replication of El-deleted HAV-5. Expression of the E1A and E1B proteins at regular intervals was also monitored by Western blot. The results indicated that the VIDO R1 line retained the ability to support the growth of E1-deleted virus and expressed similar levels of E1 proteins during more than 50 passages in culture. Therefore, VIDO R1 can be considered to be an established cell line.

Example 2: Construction of a full-length infectious clone of PAV-3.

A plasmid clone containing a full-length copy of the PAV-3 genome (pPAV-200) was generated by first constructing a plasmid containing left- and right-end sequences of PAV-3, with the PAV-3 sequences bordered by *Pac*I sites and separated by a *Pst*I restriction site (pPAV-100), then allowing recombination between *Pst*I-digested pPAV-100 and an intact PAV-3 genome. Left- and right-end sequences for insertion into pPAV-100 were produced by PCR amplification, as follows.

The plasmid p3SB (Reddy *et al.*, 1993, *Intervirology* **36**:161-168), containing the left end of PAV-3 genome (position 1-8870) was used for amplification of the first 433 bp of the PAV-3 genome by PCR. Amplification primers were oligonucleotides 1

(5'-*GCGGATCCTTAATTAA*CATCATCAATAATATACCGCACACTTTT-3') (SEQ ID NO.: 2) and 2 (5'-*CACCTGCAG*ATACACCCACACACGTCATCTCG-3') (SEQ ID NO.: 3). In the sequences shown here, adenoviral sequences are shown in bold and engineered restriction enzyme sites are italicized.

For amplification of sequences at the right end of the PAV-3 genome, the plasmid p3SA (Reddy *et al.*, 1993, *supra*) was used. This plasmid was used as template in PCR for amplification of the terminal 573 bp of the genome using oligonucleotide 1 (above) and oligonucleotide 3 (5'-*CACCTGCAG*CCTCCTGAGTGTGAAGAGTGTCC-3') (SEQ ID NO.: 4). The primers were designed based on the nucleotide sequence information described elsewhere (Reddy *et al.*, 1995*c*, *supra*; and Reddy *et al.*, 1997, *supra*).

For construction of pPAV-100, the PCR product obtained with oligonucleotides 1 and 2 was digested with *Bam*HI and *Pst*I restriction enzymes and the PCR product obtained using primers 1 and 3 was digested with *Pst*I and *Pac*I enzymes. Modified bacterial plasmid pPolyIIsnl4 was digested with *Bam*HI and *Pac*I enzymes. This plasmid was used based on its suitability for homologous recombination in *E. coli*. The two PCR products described above were cloned into pPolyIIsn14 by three way ligation to generate the plasmid pPAV-100 which carries both termini of PAV-3, separated by a *Pst*I site and bordered by *Pac*I restriction enzyme sites.

Plasmid pPAV-200, which contains a full length PAV-3 genome, was generated by co-transformation of *E. coli* BJ 5183 *recBC sbcBC* (Hanahan, 1983, *J. Mol. Biol.* **166**:557-580) with *Pst*I-linearized pPAV-100 and the genomic DNA of PAV-3. Extensive restriction enzyme analysis of pPAV-200 indicated that it had the structure expected of a full-length PAV-3 insert, and that no unexpected rearrangements had occurred during recombination in *E. coli*.

The infectivity of pPAV-200 was demonstrated by lipofectin transfection (Life Technologies, Gaithersburg, MD) of ST cells following *Pac*I enzyme digestion of the plasmid to release the viral genome from the plasmid. Viral

plaques were evident 7 days following transfection, and titers were equivalent to, or higher than, those obtained after infection with wild-type PAV. The plaques were amplified and the viral DNA was extracted and analyzed by restriction enzyme digestion. The viral DNA obtained by cleavage of pPAV-200 with *Pac*I contained an extra 3 bases at each end; but these extra bases did not substantially reduce the infectivity of the PAV genome excised from pPAV-200. In addition, the bacterial-derived genomes lacked the 55-kDa terminal protein that is covalently linked to the 5' ends of adenoviral DNAs and which enhances infectivity of viral DNA.

Example 3: Generation of E1 deletion mutants of PAV-3.

A plasmid (pPAV-101) containing the left (nucleotides 1-2,130) and the right (nucleotides 32,660-34,094) terminal *Nco*I fragments of the PAV-3 genome was constructed by digesting pPAV-200 with the enzyme *Nco*I (which has no recognition sites in the vector backbone, but many sites in the PAV insert), gel-purifying the appropriate fragment and self-ligating the ends. *See* Figure 4. The E1A sequences of pPAV-101, between nucleotides 407 and 1270 (PAV genome numbering), were deleted by digestion of pPAV-101 with *Not*I (recognition site at nucleotide 407) and *Ase*I (recognition site at 1270), generation of blunt ends, and insertion of a double-stranded oligonucleotide encoding a *Xba*I restriction site to create a plasmid, pPAV-102, containing PAV left- and right-end sequences, separated by a *Nco*I site, with a deletion of the E1A region and a *Xba*I site at the site of the deletion. *See* Figure 5. Plasmid pPAV-201, containing a full-length PAV-3 genome minus E1A sequences, was created by co-transformation of *E. coli* BJ 5183 with *Nco*I linearized pPAV-102 and genomic PAV-3 DNA . The resulting construct, when transfected into VIDO R1 cells following digestion with *Pac*I restriction enzyme, produced a virus that had a deletion in the E1 region. In similar fashion, construction of a virus with deletions in E1 and E3 was accomplished by transformation of BJ 5183 cells with *Nco*I linearized pPAV-102 and genomic PAV-3 DNA containing an E3 deletion. These

E1A deletion mutants did not grow on either ST (swine testis) cells or fetal porcine retina cells and could only be grown in the VIDO R1 cell line.

Example 4: Generation of E3 inserts and deletion mutants.

To systematically examine the extent of the E3 region that could be deleted, a E3 transfer vector was constructed. The vector (pPAV-301) contained a PAV-3 segment from nucleotides 26,716 to 31,064 with a green fluorescent protein (GFP) gene inserted into the *Sna*BI site (located at nucleotide 28,702) in the same orientation as E3. The GFP gene was obtained from the plasmid pGreen Lantern-1™ (Life Technologies), by *Not*I digestion followed by purification of a 732-nucleotide fragment. Similarly, another construct was made with GFP cloned into the *Sac*I site located at nucleotide 27,789. *Kpn*I-*Bam*HI fragments encompassing the modified E3 regions were then isolated from these E3 transfer vectors and recombined in *E coli* with pPAV-200 that had been linearized at nucleotide position 28,702 by *Sna*BI digestion. Virus were obtained with a construct that had the GFP gene cloned into the *Sna*BI site.

To delete the non-essential portion of E3 from the transfer vector, a PCR approach was used. In this approach, the region of the PAV genome between nucleotides 27,402 and 28,112 was amplified using the following primers:

5'-GACTGACGCCGGCATGCAAT-3' **SEQ ID NO: 5**

5'-CGGATCCTGACGCTACGAGCGGTTGTA-3' **SEQ ID NO: 6**

In a second PCR reaction, the portion of the PAV genome between nucleotides 28,709 and 29,859 was amplified using the following two primers:

5'-CGGATCCATACGTACAGATGAAGTAGC-3' **SEQ ID NO: 7**

5'- TCTGACTGAAGCCGACCTGC-3' **SEQ ID NO: 8**

In the oligonucleotides designated SEQ ID NO: 6 and SEQ ID NO: 7, a *Bam*HI recognition sequence is indicated by underlining. The template for amplification was a *Kpn*I-*Bam*HI fragment encompassing nucleotides 26,716-31,063 of the PAV genome, inserted into the plasmid pGEM3Z (Promega), and *Pfu* polymerase (Stratagene) was used for amplification. The first PCR product

(product of amplification with SEQ ID NO: 5 and SEQ ID NO: 6) was digested with *Bam*HI and gel- purified. The second PCR product (product of amplification with SEQ ID NO: 7 and SEQ ID NO: 8) was digested with *Bam*HI and *Spe*I and gel-purified. They were inserted into *SmaI/Spe*I-digested pBlueScript II SK(+) (Stratagene) in a three-way ligation reaction to generate pPAV-300. *See* Figure 6. pPAV-300 contains the portion of the PAV-3 genome extending from nucleotides 27,402 to 29,859, with 594 base pairs (bp) between nucleotides 28,113 and 28,707 deleted from the E3 region. A virus with such a deletion was constructed as follows. A *Sph*I-*Spe*I fragment from pPAV-300, containing part of the pVIII gene, a deleted E3 region, and part of the fiber gene was isolated (see Figure 6). This fragment was co-transfected, with *Sna*BI-digested pPAV-200 (which contains a full-length PAV-3 genome) into *E. coli*. Homologous recombination generated a plasmid, pFPAV-300, containing a full-length PAV genome with a deletion in the E3 region. pFPAV-300 was digested with *Pac*I and transfected into VIDO R1 cells (Example 1) to generate recombinant virus with a deletion in the E3 region of the genome.

Example 5: Construction of a PAV recombinant with an insertion of the PRV gp50 gene in the PAV E3 region and expression of the inserted gene

To construct a recombinant PAV expressing pseudorabies virus (PRV) gp50, the PRV gp50 gene was inserted at the *Sna*BI site of pPAV-300 to create plasmid pPAV-300-gp50. A *Sph*I-*Spe*I fragment from pPAV-300-gp50, containing part of the pVII gene, a deleted E3 region with the PRV gp50 gene inserted, and part of the fiber gene, was purified and co-transfected, along with *Sna*BI-digested pFPAV-300 (E3-deleted) into *E. coli*. In the bacterial cell, homologous recombination generated pFPAV-300-gp50, a plasmid containing a PAV genome with the PRV gp50 gene replacing a deleted E3 region. Recombinant virus particles were obtained as described in Example 4.

Expression of the inserted PRV gp50 was tested after infection of VIDO R1 cells with the recombinant virus, by ^{35}S labeling of infected cells (continuous

label), followed by immunoprecipitation with an anti-gp50 monoclonal antibody and gel electrophoresis of the immunoprecipitate. Figure 7 shows that large amounts of gp50 are present by 12 hours after infection, and expression of gp50 persists up to 24 hours after infection.

Example 6: Expression of the Chloramphenicol acetyltransferase gene from a region that lies between the promoter of the E4 region and the right ITR.

The right terminal fragment of the PAV genome (encompassing nucleotides 31,054-34,094) was obtained by *Xho*I digestion of pPAV-200 and cloned between the *Xho*I and *Not*I sites of pPolyIIsn14. A Chloramphenicol acetyltransferase (CAT) gene expression cassette, in which the CAT gene was flanked by the SV40 early promoter and the SV40 polyadenylation signal, was inserted, in both orientations, into a unique *Hpa*I site located between the E4 region promoter and the right ITR, to generate plasmids pPAV-400A and pPAV-400B. The modified terminal fragments were transferred into a plasmid containing a full-length PAV-3 genome by homologous recombination in *E coli* between the isolated terminal fragments and *Hpa*I-digested pPAV-200. Recombinant viruses expressing CAT were obtained following transfection of VIDO R1 cells with the plasmids. PAV-CAT2 contained the CAT gene cassette in a leftward transcriptional orientation (*i.e.*, the same orientation as E4 region transcription), while, in PAV-CAT6, the CAT gene cassette was in the rightward transcriptional orientation.

These recombinant viruses were tested for expression of CAT, after infection of VIDO R1 cells, using a CAT Enzyme Assay System from Promega, following the instructions provided by the supplier. *See*, Cullen (1987) *Meth. Enzymology* **43**:737; and Gorman *et al.*, (1982) *Mol. Cell. Biol.* **2**:1044. The results are shown in Table 3.

Table 3: CAT activity expressed by recombinant PAV viruses

Sample	^{3}H cpm
Mock-infected	458
CAT positive control*	199,962
PAV-CAT2	153,444
PAV-CAT6	63,386

* - the positive control sample contained 0.1 Units of purified CAT.

These results show that recombinant PAV viruses, containing an inserted gene, are viable and are capable of expressing the inserted gene.

Example 7: Construction of replication defective PAV-3 expressing GFP

A 2.3 kb fragment containing the CMV immediate early promoter, the green fluorescent protein (GFP) gene and the bovine growth hormone poly(A) signal was isolated by digesting pQBI 25 (Quantum Biotechnology) with *Bgl*II and *Dra*III followed by filling the ends with T4 DNA polymerase. This fragment was inserted into the *Srf*I site of pPAV-102 in both orientations to generate pPAV-102GFP (Figure 8). This plasmid, digested with *Pac*I and *Sma*I enzymes, and the fragment containing part of the E1 sequence and the GFP gene was gel purified. This fragment and the SrfI digested pFPAV-201 were used to transform *E. coli* BJ 5183 to generate the full-length clone containing GFP in the E1 region (pFPAV-201-GFP) by homologous recombination. The recombinant virus, PAV3delE1E3.GFP was generated following transfection of VIDO R1 cells with *Pac*I restricted pFPAV-201-GFP that had the GFP transcription unit in the opposite orientation to the E1. A similar virus with the GFP in the same orientation as E1 could not be rescued from transfected cells. Presence of the GFP gene in the viral genome was confirmed by restriction enzyme analysis. The recombinant virus replicated in VIDO R1 cells two logs less efficiently than the wild type PAV-3.

Example 8: Virus entry and replication of PAV-3 in human and animal cells.

To initially characterize the host species restriction of PAV *in vitro,* monolayers of 11 cell types from 6 different mammalian species were infected with wild type PAV-3 or PAV3del.E1E3.GFP. ST, VIDO R1 (porcine), 293, A549 (human), MDBK, VIDO R2 (bovine), C3HA (mouse), COS, VERO (monkey), sheep skin fibroblasts or cotton rat lung cells were incubated with 1 pfu/cell of wild type PAV-3 or helper-dependent PAV-3 expressing GFP. The cells infected with wild type PAV were harvested at 2 h and 3 days post-infection, subjected to two cycles of freeze-thaw, and virus titers were determined on VIDO R1 cells. Cells that were infected with the recombinant virus expressing GFP were observed with the aid of a fluorescent microscope for green fluorescence.

A ten-fold increase in virus titers in Vero and COS cells, and a hundred-fold increase in cotton rat lung fibroblasts and VIDO R2 cells, was noticed. No increase in the virus titers was observed with 293, A549, MDBK, sheep skin fibroblasts, dog kidney and C3HA cells. All of these cell types showed bright green fluorescence when infected with PAV3delE1E3.GFP except human cells, which showed a weak fluorescence. In addition, low levels of GFP expression were achieved in human cells with recombinant PAV-3. These observations suggest that virus entry into human cells is limited and/or the human cells are non-permissive for the replication of the virus. These results also demonstrated that GFP was expressed by the PAV-3 vector in cells which are semi-permissive (VERO, COS, Cotton rat lung fibroblasts and VIDO R2), or non-permissive (Sheep skin fibroblasts, MDBK and human cells) for virus replication.

Example 9: Insertions in the regions of the PAV-3 genome defined by nucleotides 145-13,555; 15,284-19,035; 22,677-24,055; 26,573-27,088; and 31,149-34,094

Insertions are made by art-recognized techniques including, but not limited to, restriction digestion, nuclease digestion, ligation, kinase and phosphatase

treatment, DNA polymerase treatment, reverse transcriptase treatment, and chemical oligonucleotide synthesis. Heterologous nucleic acid sequences of interest are cloned into plasmid vectors containing portions of the PAV genome (which may or may not contain deletions of PAV sequences) such that the foreign sequences are flanked by sequences having substantial homology to a region of the PAV genome into which insertion is to be directed. Substantial homology refers to homology sufficient to support homologous recombination. These constructs are then introduced into host cells that are co-transfected with PAV-3 DNA or a cloned PAV genome. During infection, homologous recombination between these constructs and PAV genomes will occur to generate recombinant PAV genome-containing plasmids. Recombinant virus are obtained by transfecting the recombinant PAV genome-containing plasmids into a suitable mammalian host cell line. If the insertion occurs in an essential region of the PAV genome, the recombinant PAV virus is propagated in a helper cell line which supplies the viral function that was lost due to the insertion.

Deposit of Biological Materials

The following materials were deposited and are maintained with the Veterinary Infectious Disease Organization (VIDO), Saskatoon, Saskatchewan, Canada.

The nucleotide sequences of the deposited materials are incorporated by reference herein, as well as the sequences of the polypeptides encoded thereby. In the event of any discrepancy between a sequence expressly disclosed herein and a deposited sequence, the deposited sequence is controlling.

Material	Internal Accession No.	Deposit Date
Recombinant plasmids		
pPAV-101	VIDO 98-1	April 10, 1998
pPAV-102	VIDO 98-2	April 10, 1998
pPAV-200	VIDO 98-3	April 10, 1998
pPAV-300	VIDO 98-4	April 10, 1998
pPAV-400A	VIDO 98-5	April 10, 1998
pPAV-400B	VIDO 98-6	April 10, 1998
Recombinant cell lines		
Porcine embryonic retinal cells transformed with HAV-5 E1 sequences:		
VIDO R1	VIDOO 98C-1	April 10 1998

While the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that various changes and modifications may be practiced without departing from the spirit of the invention. Therefore the foregoing descriptions and examples should not be construed as limiting the scope of the invention.

CLAIMS

What is claimed is:

1. A nucleotide sequence that is substantially homologous to the genome of porcine adenovirus type 3 (PAV-3), or a fragment thereof.

2. A nucleotide sequence that is substantially homologous to a portion of the genome of porcine adenovirus type 3, said portion selected from the group consisting of nucleotides 145-13,555; 15,284-19,035; 22,677-24,055; 26,5732-27,088; and 31,149-34,094 of the PAV-3 genome, or a fragment thereof.

3. A nucleotide sequence that is substantially homologous to a PAV-3 gene or fragment thereof, wherein the gene is selected from the group consisting of E1A, E1B, E4, pIX, DBP, pTP, pol, IVa2, 52K, IIIA, pIII, pVII, pV, pX, pVI, and 33K.

4. A nucleotide sequence according to claim 3 comprising a PAV-3 coding sequence from a region of the PAV-3 genome selected from the group consisting of E1A, E1B, E4, pIX, DBP, pTP, pol, IVa2, 52K, IIIA, pIII, pVII, pV, pX, pVI, and 33K, and fragments thereof.

5. A nucleotide sequence that is substantially homologous to the nucleotide sequence of claim 4.

6. A nucleotide sequence comprising an intergenic region of the PAV-3 genome.

7. A nucleotide sequence that is substantially homologous to the nucleotide sequence of claim 6.

8. A nucleotide sequence encoding a PAV-3 regulatory sequence.

9. The nucleotide sequence of claim 8, wherein the regulatory sequence is selected from the group consisting of a transcriptional regulatory sequence, a promoter, an enhancer, an upstream regulatory domain, a splicing signal, a polyadenylation signal, a transcriptional termination sequence, a translational regulatory sequence, a ribosome binding site and a translational termination sequence.

10. A vector comprising the nucleotide sequence of claim 1.

11. A vector comprising the nucleotide sequence of claim 2.

12. A vector comprising the nucleotide sequence of claim 3.

13. A vector comprising the nucleotide sequence of claim 8.

14. A defective recombinant PAV vector, comprising ITR sequences, packaging sequences, and at least one heterologous nucleotide sequence, wherein the PAV vector lacks E1 function.

15. A defective recombinant PAV vector according to claim 14, wherein the vector is deleted in the E1 region.

16. A defective recombinant PAV vector according to claim 15, wherein the vector is additionally deleted in a region selected from the group consisting of E2, E3, E4, L1, L2, L3, L4, L5, L6 and the region between E4 and the right end of the genome.

17. A defective recombinant PAV vector according to claim 16, wherein the vector is additionally deleted in more than one region selected from the group consisting of E2, E3, E4, L1, L2, L3, L4, L5, L6 and the region between E4 and the right end of the genome.

18. A defective recombinant PAV vector according to claim 14, wherein the heterologous nucleotide sequence encodes a therapeutic polypeptide.

19. A defective recombinant PAV vector according to claim 18, wherein the therapeutic polypeptide is selected from the group consisting of coagulation factors, growth hormones, cytokines, lymphokines, tumor-suppressing polypeptides, cell receptors, ligands for cell receptors, protease inhibitors, antibodies, toxins, immunotoxins, dystrophins, cystic fibrosis transmembrane conductance regulator (CFTR) and immunogenic polypeptides.

20. A recombinant PAV vector comprising a PAV genome and at least one heterologous nucleotide sequence, wherein the heterologous sequence is inserted in a region selected from the group consisting of the E1 region, the E3 region, the E4 region and the region between E4 and the right end of the genome.

21. The recombinant PAV vector of claim 20, comprising two or more heterologous nucleotide sequences.

22. The recombinant PAV vector of claim 21, wherein the two or more heterologous nucleotide sequences are inserted at different sites.

23. The recombinant PAV vector of claim 20, wherein the heterologous nucleotide sequence encodes a therapeutic polypeptide.

24. The recombinant PAV vector of claim 23, wherein the therapeutic polypeptide is selected from the group consisting of coagulation factors, growth hormones, cytokines, lymphokines, tumor-suppressing polypeptides, cell receptors, ligands for cell receptors, protease inhibitors, antibodies, toxins, immunotoxins, dystrophins, cystic fibrosis transmembrane conductance regulator (CFTR) and immunogenic polypeptides.

25. A method for obtaining a recombinant PAV comprising a heterologous nucleotide sequence inserted into an insertion site, the method comprising the steps of:

(a) providing a PAV genome;

(b) providing a heterologous nucleotide sequence;

(c) linking the heterologous nucleotide sequence to guide sequences, the guide sequences being substantially homologous to PAV sequences flanking the insertion site, such that guide sequences are present at both ends of the heterologous sequence;

(d) introducing the construct from step (c) into a cell together with the PAV genome;

(e) allowing homologous recombination to occur between the two sequences from step (d) to generate a recombinant PAV genome;

(f) purifying the recombinant PAV genome;

(g) inserting the recombinant PAV genome into a mammalian cell;

(h) culturing the mammalian cell under conditions wherein the recombinant PAV genome is replicated and packaged; and

(i) collecting the recombinant PAV from the cell or the culture medium.

26. The method according to claim 25 wherein the insertion site is located in a region of the PAV genome selected from the group consisting of the E1 region, the E3 region, the E4 region and the region between E4 and the right end of the genome.

27. The method according to claim 26 wherein the PAV genome is deleted in a region selected from the group consisting of the E1 region, the E3 region, the E4 region and the region between E4 and the right end of the genome.

28. A method for producing a recombinant PAV that comprises introducing the PAV vector of claim 14 into an appropriate helper cell line and recovering virus from the infected cells.

29. The method according to claim 28 wherein the helper cell line provides E1 function.

30. A host cell comprising the vector of claim 10.

31. A host cell comprising the vector of claim 11.

32. A host cell comprising the vector of claim 12.

33. A host cell comprising the vector of claim 13.

34. A host cell comprising the vector of claim 14.

35. A host cell comprising the vector of claim 20.

36. A recombinant porcine cell line comprising an adenovirus E1 gene region, said recombinant cell line thereby providing E1 function and allowing replication of a PAV vector having a deletion in the E1 region.

37. A method for producing a recombinant PAV, the method comprising:

(a) introducing, into the cell line of claim 36, a vector comprising ITR sequences, PAV packaging sequences, and at least one heterologous nucleotide sequence, wherein the vector is deleted in the E1 region and the vector is additionally deleted in one or more regions selected from the group consisting of the E2 region, the E3 region, the E4 region, and the region between E4 and the right end of the genome;

(b) culturing the cell line under conditions whereby virus replication and packaging occurs; and

(c) recovering virus from the infected cells.

38. A method for producing a recombinant PAV polypeptide, the method comprising:

(a) providing a population of host cells according to claim 31; and

(b) growing said population of cells under conditions whereby the PAV polypeptide is expressed.

39. A method for producing a recombinant PAV polypeptide, the method comprising:

(a) providing a population of host cells according to claim 32; and

(b) growing said population of cells under conditions whereby the PAV polypeptide is expressed.

40. A method for producing a recombinant polypeptide, the method comprising:

(a) providing a population of host cells according to claim 33, and

(b) growing said population of cells under conditions whereby the polypeptide is expressed.

41. A method for producing a recombinant polypeptide, the method comprising:

(a) providing a population of host cells according to claim 34, and

(b) growing said population of cells under conditions whereby the polypeptide is expressed.

42. A method for producing a recombinant polypeptide, the method comprising:

(a) providing a population of host cells according to claim 35, and

(b) growing said population of cells under conditions whereby the polypeptide is expressed.

43. A PAV polypeptide encoded by the nucleotide sequence of claim 2.

44. A PAV polypeptide encoded by the nucleotide sequence of claim 3.

45. A PAV polypeptide produced by the method of claim 38.

46. A PAV polypeptide produced by the method of claim 39.

47. A recombinant polypeptide produced by the method of claim 40.

48. A recombinant polypeptide produced by the method of claim 41.

49. A recombinant polypeptide produced by the method of claim 42.

50. A method for detecting the presence of PAV nucleotide sequences in a biological sample comprising:

(a) providing a biological sample; and

(b) subjecting the sample to hybridization using a labeled probe comprising at least ten continuous nucleotides of the sequence according to claim 1.

51. A method for detecting the presence of PAV nucleotide sequences in a biological sample comprising:

(a) providing a biological sample; and

(b) subjecting the sample to hybridization using a labeled probe comprising at least ten continuous nucleotides of the sequence according to claim 3.

52. A method for detecting the presence of PAV nucleotide sequences in a biological sample comprising:

(a) providing a biological sample; and

(b) subjecting the sample to a polymerase chain reaction using primers comprising at least ten continuous nucleotides of the sequence according to claim 1.

53. A method for detecting the presence of PAV nucleotide sequences in a biological sample comprising:

(a) providing a biological sample; and

(b) subjecting the sample to a polymerase chain reaction using primers comprising at least ten continuous nucleotides of the sequence according to claim 3.

54. A kit for detecting the presence of PAV nucleotide sequences in a biological sample comprising one or more probes, said probes comprising at least ten continuous nucleotides of the sequence according to claim 1.

55. A kit for detecting the presence of PAV nucleotide sequences in a biological sample comprising one or more probes, said probes comprising at least ten continuous nucleotides of the sequence according to claim 3.

56. A method for detecting the presence of PAV antibodies in a biological sample comprising:

(a) providing a biological sample; and

(b) subjecting the sample to immunoassay using the polypeptide of claim 43.

57. A method for detecting the presence of PAV antibodies in a biological sample comprising:

(a) providing a biological sample; and

(b) subjecting the sample to immunoassay using the polypeptide of claim 44.

58. A method for detecting the presence of PAV antibodies in a biological sample comprising:

(a) providing a biological sample; and

(b) subjecting the sample to immunoassay using the polypeptide of claim 45.

59. A method for detecting the presence of PAV antibodies in a biological sample comprising:

(a) providing a biological sample; and

(b) subjecting the sample to immunoassay using the polypeptide of claim 46.

60. A kit for detecting the presence of PAV antibodies in a biological sample comprising one or more polypeptides according to claim 43.

61. A kit for detecting the presence of PAV antibodies in a biological sample comprising one or more polypeptides according to claim 44.

62. A kit for detecting the presence of PAV antibodies in a biological sample comprising one or more polypeptides according to claim 45.

63. A kit for detecting the presence of PAV antibodies in a biological sample comprising one or more polypeptides according to claim 46.

64. A method for obtaining antibodies to PAV, the method comprising:

(a) providing a PAV polypeptide according to claim 43;

(b) immunizing a mammalian host with the polypeptide; and

(c) collecting antibody from the immunized host.

65. A method for obtaining antibodies to PAV, the method comprising:

(a) providing a PAV polypeptide according to claim 44;

(b) immunizing a mammalian host with the polypeptide; and

(c) collecting antibody from the immunized host.

66. A method for obtaining antibodies to PAV, the method comprising:

(a) providing a PAV polypeptide according to claim 45;

(b) immunizing a mammalian host with the polypeptide; and

(c) collecting antibody from the immunized host.

67. A method for obtaining antibodies to PAV, the method comprising:

(a) providing a PAV polypeptide according to claim 46;

(b) immunizing a mammalian host with the polypeptide; and

(c) collecting antibody from the immunized host.

68. A method for detecting the presence of a PAV antigen in a biological sample, the method comprising:

(a) providing a biological sample; and

(b) subjecting the sample to immunoassay using an antibody according to claim 64.

69. A method for detecting the presence of a PAV antigen in a biological sample, the method comprising:

(a) providing a biological sample; and

(b) subjecting the sample to immunoassay using an antibody according to claim 65.

70. A method for detecting the presence of a PAV antigen in a biological sample, the method comprising:

(a) providing a biological sample; and

(b) subjecting the sample to immunoassay using an antibody according to claim 66.

71. A method for detecting the presence of a PAV antigen in a biological sample, the method comprising:

(a) providing a biological sample; and

(b) subjecting the sample to immunoassay using an antibody according to claim 67.

72. A method for detecting the presence of an antigen in a biological sample comprising:

(a) providing a biological sample; and

(b) subjecting the sample to immunoassay using the polypeptide of claim 47.

73. A method for detecting the presence of an antigen in a biological sample comprising:

(a) providing a biological sample; and

(b) subjecting the sample to immunoassay using the polypeptide of claim 48.

74. A method for detecting the presence of an antigen in a biological sample comprising:

(a) providing a biological sample; and

(b) subjecting the sample to immunoassay using the polypeptide of claim 49.

75. A kit for detecting the presence of an antigen in a biological sample comprising one or more polypeptides according to claim 47.

76. A kit for detecting the presence of an antigen in a biological sample comprising one or more polypeptides according to claim 48.

77. A kit for detecting the presence of an antigen in a biological sample comprising one or more polypeptides according to claim 49.

78. A pharmaceutical composition capable of inducing an immune response in a mammalian subject, said composition comprising a nucleotide sequence according to claim 2.

79. A pharmaceutical composition capable of inducing an immune response in a mammalian subject, said composition comprising a nucleotide sequence according to claim 3.

80. A pharmaceutical composition capable of inducing an immune response in a mammalian subject, said composition comprising a polypeptide according to claim 43.

81. A pharmaceutical composition capable of inducing an immune response in a mammalian subject, said composition comprising a polypeptide according to claim 44.

82. A pharmaceutical composition capable of inducing an immune response in a mammalian subject, said composition comprising a polypeptide according to claim 45.

83. A pharmaceutical composition capable of inducing an immune response in a mammalian subject, said composition comprising a polypeptide according to claim 46.

84. A pharmaceutical composition capable of inducing an immune response in a mammalian subject, said composition comprising a polypeptide according to claim 47.

85. A pharmaceutical composition capable of inducing an immune response in a mammalian subject, said composition comprising a polypeptide according to claim 48.

86. A pharmaceutical composition capable of inducing an immune response in a mammalian subject, said composition comprising a polypeptide according to claim 49.

87. A pharmaceutical composition comprising the defective recombinant PAV vector of claim 14.

88. A pharmaceutical composition capable of inducing an immune response in a mammalian subject, said composition comprising a defective recombinant PAV vector according to claim 87, wherein the vector encodes an immunogenic polypeptide.

89. A pharmaceutical composition comprising the vector of claim 10.

90. A pharmaceutical composition comprising the vector of claim 11.

91. A pharmaceutical composition comprising the vector of claim 12.

92. A pharmaceutical composition comprising the vector of claim 13.

93. A pharmaceutical composition comprising the vector of claim 20.

94. A pharmaceutical composition comprising a recombinant PAV obtained according to the method of claim 25.

95. A pharmaceutical composition comprising a recombinant PAV obtained according to the method of claim 37.

96. A pharmaceutical composition comprising the host cell of claim 30.

97. A pharmaceutical composition comprising the host cell of claim 31.

98. A pharmaceutical composition comprising the host cell of claim 33.

99. A method for eliciting an immune response in a mammalian host to protect against an infection comprising administering a pharmaceutical composition comprising a recombinant PAV vector capable of expressing a protective antigen.

100. A method of gene therapy in a mammalian host comprising administering to the host the vector of claim 11.

101. A method of gene therapy in a mammalian host comprising administering to the host the vector of claim 13.

102. A method of gene therapy in a mammalian host comprising administering to the host the defective recombinant PAV vector of claim 14.

103. A method of gene therapy in a mammalian host comprising administering to the host the recombinant PAV vector of claim 20.

104. A method of gene therapy in a mammalian host comprising administering to the host a recombinant PAV vector obtained according to the method of claim 25.

105. A method of gene therapy in a mammalian host comprising administering to the host a recombinant PAV obtained according to the method of claim 37.

106. A method of gene therapy in a mammalian subject comprising administering to the subject the host cell of claim 31.

107. A method of gene therapy in a mammalian subject comprising administering to the subject the host cell of claim 32.

108. A method of gene therapy in a mammalian subject comprising administering to the subject the host cell of claim 33.

109. A method of gene therapy in a mammalian subject comprising administering to the subject the host cell of claim 34.

110. A method of gene therapy in a mammalian subject comprising administering to the subject the host cell of claim 35.

111. An antibody that specifically binds the polypeptide of claim 43.

112. An antibody that specifically binds the polypeptide of claim 44.

113. An antibody that specifically binds the polypeptide of claim 45.

114. An antibody that specifically binds the polypeptide of claim 46.

115. A composition comprising a full-length genomic clone of a PAV genome.

116. The composition of claim 115 wherein the PAV genome is the genome of PAV-3.

117. A method for obtaining a full-length genomic clone of a PAV genome, the method comprising:

(a) providing two or more cloned segments of the PAV genome, wherein the cloned segments of the PAV genome, taken together, represent the entire PAV genome;

(b) introducing the two or more cloned segments of the PAV genome into a cell;

(c) allowing homologous recombination to occur within the cell between the two or more cloned segments of the PAV genome to generate a full-length PAV genome; and

(d) purifying the full-length PAV genome from the cell.

118. A full-length PAV genome produced by the method of claim 117.

CATCATCAATAATATACCGCACACTTTTATTGCCCCTTTTGTGGCGTGGTGATTGGCGGAGAGGGT
TGGGGGCGGCGGGCGGTGATTGGTGGAGAGGGGTGTGACGTAGCGTGGGAACGTGACGTCGCGTGG
GAAAATGACGTGTGATGACGTCCCGTGGGAACGGGTCAAAGTCCAAGGGGAAGGGGTGGAGCCCTG
GGGCGGTCCTCCGCGGGGCGGGGCCGAGCGGCGGAAATTCCCGCACAGGTGGAGAGTACCGCGGGA
TTTTGTGCCCTCTGGACCGGACCTTCGCCCTCCGGTGTGGCACTTCCGCACCACACGTCCGCGGCC
CGGTATTCCCCACCTGACGACGGTGACACCACTCACCTGAGCGGGGTGTCCTTCGCGCTGAGAGGT
CCGCGGCGGCCGCCCGAGATGACGTGTGTGGGTGTATTTTTTCCCCTCAGTGTATATAGTCCGCGC
AGCGCCCGAGAGTCACTACTCTTGAGTCCGAAGGGAGTAGAGTTTTCTCTCAGCGGAACAGACCCT
CGACATGGCGAACAGACTTCACCTGGACTGGGACGGAAACCCCGAGGTGGTGCCGGTGCTGGAATG
GGACCCGGTGGATCTGCGCGACCCCTCTCCGGGGGGATGAGGGCTTCTGTGAGCCGTGCTGGGAGAG
TCTGGTCGATGGACTGCCGGACGAGTGGCTGGACAGTGTGGACGAGGTGGAGGTGATTGTGACTGA
GGGGGGTGAGTCAGAGGACAGTGGTGGGAGTGCCGCTGGTGACTCAGGTGGCTCTCAGGGGGTCTT
TGAGATGGACCCCCCAGAAGAGGGGGACAGTAATGAGGAGGATATCAGCGCGGTGGCTGCGGAGGT
GCTGTCTGAACTGGCTGATGTGGTGTTTGAGGACCCACTTGCGCCACCCTCTCCGTTTGTGTTGGA
CTGCCCCGAGGTACCTGGTGTGAACTGCCGCTCTTGTGATTACCATCGCTTTCACTCCAAGGACCC
CAATCTGAAGTGCAGTCTGTGCTACATGAGGATGCATGCCTTTGCTGTCTATGGTGAGTGTTTTTG
GACATTTGTGGGATTATGTGGAAAAAAAGGAAAAAGTGCTTGTAAGAAATCTCATGTGCTATTTCC
CATTTTTTGTCTTTTTAGAAGCTGTTTCTCCAGCACCTCACAGGTCGGGTTCCCCGGGACTTGGAG
ACCTGCCAGGACGCAAGAGGAAGTACTGCTATGACTCATGCAGCGAACAACCTTTGGACCTGTCTA
TGAAGCGCCCCCGCGATTAATCATTAACCTCAATAAACAGCATGTGATGATGACTGATTGTCTGTG
TCTCTGCCTATATATACCCTTGTGGTTTGCAGGGAAGGGATGTGGTGACTGAGCTATTCCTCAGCA
TCATCATCGCTCTGCTTTTTTCTACTGCAGGCTATTTCTTGCTAGCTCGCTGTCCCTTTTCTTTTT
CTGTGGGCATGGACTATCAACTTCTGGCCAAGCTTACTAACGTGAACTACCTTAGGAAGGTGATAG
TACAGGGGTCTCAGAACTGCCCTTGGTGGAAAAAGATTTTTTCGGACAGGTTTATCAAGGTAGTAG
CAGAGGCCAGGAGGCAGTACGGGCAAGAGTTGATTGAGATTTTTGTGGAGGGTGAGAGGGGCTTTG
GTCCTGAGTTCCTGCGGGAAGGGGGACTGTACGAAGAGGCCGTTCTGAAAGAGTTGGATTTCAGCA
CCTTGGGACGCACCGTAGCTAGTGTGGCTCTGGTCTGCTTCATTTTTGAGAAGCTTCAGAAGCACA
GCGGGTGGACTGACGAGGGTATTTTAAGTCTTCTGGTGCCGCCACTATGTTCCCTGCTGGAGGCGC
GAATGATGGCGGAGCAGGTGCGGCAGGGGCTGTGCATCATCAGGATGCCGAGCGCGGAGCGGGAGA
TGCTGTTGCCCAGTGGGTCATCCGGCAGTGGCAGCGGGGCCGGGATGCGGGACCAGGTGGTGCCCA
AGCGCCCGCGGGAGCAGGAAGAGGAGGAGGAGGACGAGGATGGGATGGAAGCGAGCGGGCGCAGGC
TCGAAGGGCCGGATCTGGTTTAGATCGCCGCCGGCCCGGGGGAGCGGGTGGAGAGGGGAGCGGGGA
GGAGGCGGGGGGGTCTTCCATGGTTAGCTATCAGCAGGTGCTTTCTGAGTATCTGGAGAGTCCTCT
GGAGATGCATGAGCGCTACAGCTTTGAGCAGATTAGGCCCTATATGCTTCAGCCGGGGGATGATCT
GGGGGAGATGATAGCCCAGCACGCCAAGGTGGAGTTGCAGCCGGGCACGGTGTACGAGCTGAGGCG
CCCGATCACCATCCGCAGCATGTGTTACATCATCGGGAACGGGGCCAAGATCAAGATTCGGGGGAA
TTACACGGAGTACATCAACATAGAGCCGCGTAACCACATGTGTTCCATTGCGGGCATGTGGTCGGT
GACTATCACGGATGTGGTTTTTGATCGGGAGCTACCGGCCCGGGGTGGTCTGATTTTAGCCAACAC
GCACTTCATCCTGCACGGCTGCAACTTCCTGGGCTTTCTGGGCTCGGTAATAACGGCGAACGCCGG
GGGGGTGGTGCGGGGATGCTACTTTTTCGCCTGCTACAAGGCGCTGGACCACCGGGGGCGGCTGTG
GCTGACGGTGAACGAGAACACGTTTGAAAAGTGTGTGTACGCGGTGGTCTCTGCGGGGCGTTGCAG
GATCAAGTACAACTCCTCCCTGTCCACCTTCTGCTTCTTGCACATGAGCTATACGGGCAAGATAGT
GGGGAACAGCATCATGAGCCCTTACACGTTCAGCGACGACCCCTACGTGGACCTGGTGTGCTGCCA
GAGCGGGATGGTGATGCCCCTGAGCACGGTGCACATCGCTCCCTCGTCTCGCCTGCCCTACCCTGA
GTTCCGCAAGAATGTGCTCCTCCGCAGCACCATGTTTGTGGGCGGCCGCCTGGGCAGCTTCAGCCC
CAGCCGCTGCTCCTACAGCTACAGCTCCCTGGTGGTGGACGAGCAGTCCTACCGGGGTCTGAGTGT
GACCTGCTGCTTCGATCAGACCTGTGAGATGTACAAGCTGCTGCAGTGTACGGAGGCGGACGAGAT
GGAGACGGATACCTCTCAGCAGTACGCCTGCCTGTGCGGGGACAATCACCCCTGGCCGCAGGTGCG
GCAGATGAAAGTGACAGACGCGCTGCGGGCCCCCCGGTCCCTGGTGAGCTGCAACTGGGGGGAGTT
CAGCGATGACGATGACTGAGGATGAGTCACCCCCTCCCCTCCTCTTGCAGGTACGTGGCCCCGCCC
AGTGGGATGGGCTTTGGATGGGGGAGGGGTGTTCCCTATAAAAGGGGGATGGGGGTGGAGGCATGC
AGCCCCACGGGGAAGCTTGTGTGGAGGATGTCTTCCGAGGGTGAGATCCGGACCTGCTTCATTTCA

FIG._1-1

GCTCGTCTTCCCAGCTGGGCCGGCGTGCGTCAGGGAGTGGCCGGGACGAATGTGAACGGCGGAGTG
GTGGGCGCCCCTGCCCAGAGCGGGGTGCTGGCCTACTCCCGCTTCGTTCAGCAGCAACAGCAGCAG
CCGGGGACGGCGGCGACGGGGTCTGTGTTCCGGGCGGTGTTTCCATCGGTGGATCTGAGCGCGGAG
GTGGGCATGATGCGGCAGGCGCTGGCGGAGCTGCGGCAGCAGCTGCAGGAGCTGCGGGAGGTGGTG
GAGATACAGCTGCGGGCCACGGCCTCGGAGGCGGCCGAGGAGGAAGAGGAGGAGGAGATTGTGGTG
GACGAGGAGGTGGCGCCCGGCGCTGGAGCGAACACCATGGAAGAGGAGGAGGATGAGATGGTCCTG
ACGATGACTGTGGTGGGGGACCCTGAGCCTGCTGGAGTGGAAGCCCAGCCGCCACCACCACCCACC
CCGGAGAGCGACCCTGCGGTGCCTGCTACTACCACTACCCCGAAGCGGCTCAGCTACGGCGCGAGC
AAGAGGAGCGGTCCATGCGCGGAGGACAACTGACGCGGACTGTGGGGGGAAGAAGGGGGAGGAGGA
AAGAAGACCATGGAGACGGGTGTTTGTCTTTTTCCAGCCCAACTTTATTGAGAATAATAATAAAGC
TTATGGATGTTTGGAACGATAATAGCGTGTCCAGCGTTCTCTGTCTTGCAGGGTCTTGTGTATCTT
CTCGAGGCACCGGTAGACCTGGTGTTGGACGTTGAAATACATGGGCATGACTCCCTCGGCGGGGTG
CAGGTAAAGCCACTGGAGGGCTGGGTGCGGGGGGCAGGTGCAGTAGATGATCCAGTCATAGGCGTT
CTGGTTGCGGTGGTGGTTGAAAATGTCCTTGAGGAGCAGGCTGATGGCGGTGGGCAGACCCTTGGT
GTAGGCATTGATGAACCGGTTGACCTGGGCGGGCTGCATGAGGGGGGACATGATGTGGTACTTGGC
CTGGATCTTGAGGTTGGAGATGTTGCCGCTCTGGTCGCGGCGGGGGTTCATGTTGTGGAGGACGAC
GAGGACGGCGTAGCCGGTGCAGCGGGGGAAGCGGGCGTGCAGCTTGGAGGGGAAGGCGTGGAAGAA
CTTGGCGACCCCCTTGTGTCCGCCGAGGTCCTCCATGCACTCGTCGAGGACGATGGCGATGGGTCC
GCGGGCGGCGGCGCGGGCGAAGACGTTGCGTGAGTCAGTGACATCATAGTTGTGCTCCTGCATGAG
GTCCTGGTAGCTCATGCGGACAAAGTCTGGCATGAGGGTGGCGGTCTGGGGGATTAGGGTGTGGTC
CGGACCGCTGCGGTAGTTGCCCTCGCAGATCTGGGTCTCCCAGGCGACTACCTCCTGCGGGGGGAT
CATGTCCACCTGCGGGGTGATGAAGAAAACAGTCTCCGGCGGGGGGGAGAGGAGTTGGGAGGAGAT
GAGGTTGCGGAGCAGCTGGGACTTGCCGGAGCCGGTGGGACCGTAGATGACAGCGATGACTGGCTG
GACCTGGTAGTTGAGGGAGCGGCAGGTGCCAGCCGGGGTGAGGAAGGGCATGCAGGCGTTGAGGGT
GTCGCGCAGGTTGCGGTTCTCTTGGACGAGGTCCTGCAGGAGGTGTCGGCCTCCCAGGGAGAGGAG
GTGGGAGAGGGAGGCGAAGGCCTTGAGGGGCTTGAGGCCCTCGGCGTAGGGCATGTCCTGCAGGGC
CTGGTGGAGCACGCGCATGCGCTCCCAGAGCTCGGTTACATGTCCCACGGTATCGTCCTCCAGCAG
GTCTGGTTGTTTCTCGGGTTGGGGTTGCTGCGTGAGTACGGAACGAGGCGGTGGGCGTCGAGCGGG
TGGAGGGTCCGGTCCTTCCAGGGCCGGAGGGCCCGCGTGAGGGTGGTCTCGGTGACGGTGAAGGGG
GCGGTCTGGGGCTGCTCGGTGGCCAGGGTCCTCTTGAGGCTGAGGCGGCTGGTGCTGAAGGTGGCG
CTTCCGAGCTGCGCGTCGTTCAGGTAGCACTGGCGGAGGAGGTCATAGGAGAGGTGTTGGGTGGCA
TGGCCCTTGGCGCGGAGCTTGCCGGGGCCGCGGTGCCCGCAAGCATCGCAAACGGTGTCGCGCAGG
GCGTAGAGCTTGGGGGCGAGCAGGACCGTCTCGGAGCTGTGGGCGTCGCTGCGGCAGCGCTCGCAC
TGGGTCTCGCACTCGACCAGCCAGGTGAGCTGGGGGTTCTGGGGATCGAAGACGAGGGGGCCCCCG
TTCCGCTTGAGGCGGTGTTTACCTTTGGTCTCCATGAGCTCGCGTCCGGCGCGGGTGAGGAAGAGG
CTGTCGGTGTCCCCGTAGACGGAGCGCAGGGGCCGGTCGGCGATGGGGGTGCCGCGGTCGTCGGCG
TAGAGGATGAGGGCCCACTCGGAGATGAAGGCACGCGCCCAGGCGAGGACGAAGCTGGCGACCTGC
GAGGGGTAGCGGTCGTTGGGCACTAATGGCGAGGCCTGCTCGAGCGTGTGGAGACAGAGGTCCTCG
TCGTCCGCGTCCAGGAAGTGGATTGGTCGCCAGTGGTAGTCCACGTGACCGGCTTGCGGGTCGGGG
GGTATAAAAGGCGCGGGCCGGGGTGCGTGGCCGTCAGTTGCTTCGCAGGCCTCGTCACCGGAGTCC
GCGTCTCCGGCGTCTCGCGCTGCGGCTGCATCTGTGGTCCCGGAGTCTTCAGGTGGGTACGCTACG
ACAAAGTCCGGGGTGACCTCAGCGCTGAGGTTGTCTGTTTCTATGAAGGCGGAGGAGCGGACGGAG
AGGTCGCCGCGGGCGATGGCTTCGGTGGTGCGGGCGTCCATCTGGCTGGCGAAGACCACCTTCTTA
TTGTCGAGGCGTGTGGCGAAACTGCCGTAGAGGGCGTTGGAGAGAAGCTTGGCGATGCTGCGGAGC
GTTTGGTTTCTGTCCCGGTCGGCCTTTTCCTTGGCAGCGATGTTGAGCTGCACGTAGTCTCGGGCG
AGGCAGCGCCACTCGGGGAAGATGCTGTTGCGCTCGTCCGGCAGGAGGCGCACGGCCCAGCCACGG
TTGTGGAGGGTGACCACGTCCACGGAGGTGGCTACCTCGCCGCGGAGGGGCTCGTTGGTCCAGCAG
AGGCGGCCGCCCTTGCGGGAGCAGTAGGGGGGCAGGACGTCCAGCTGGTCCTCGTCGGGGGGGTCG
GCGTCGATGGTGAAGAGGGCGGGCAGGAGGTCGGGGTCGAAGTAGCTGAGGGGCTCGGGGCCGTCG
AGGCGGTCCTGCCAGCGGCGGGCGGCCAGGGCGCGGTCGAAGGGGTTGAGGGGTTGGCCGGCGGGG
AAGGGGTGGGTGAGGGCGCTGGCATACATGCCGCAGATGTCATAGACGTAGAGGGGCTCCCGCAGG
AGGCCGATGAAGTTGGGGTAGCAGCGGCCGCCGCGCAGGCTCTTCGCGGACGTAGTCATACAGCTC
GTGGGAGGGCGCGAGGAGGTTCGGCCGAGGTGCGGCGCCTGGGGCCGGCTGGCGCGGTAGAGGAGC
TGCTTGAAGATGGCGTGGGAGTTGGAGCTGATGGTGGGCCTCTGGAAGACATTGAAGGCGGCGTGG
GGAAGGCCGGCCTGCGTGTGGACGAAGGCGCGGTAGGACTCTTGCAGCTTGCGGACCAGACGGGCG
GTGACGACGACGTCCTGGGCGCAGTAGCGCAGGGTGGCCTGGACGATGTCGTAAGCGTCCCCCTGG

FIG._1-2

CTCTCCTTCTTCCACAGGTCCTTGTTGAGGAGGTACTCCTGATCGCTGTCCCAGTACTTGGCGTGT
GGGAAGCCGTCCTGATCGCGTAAGTAGTCCCCCGTGCGGTAGAACTCGTTCACGGCATCGTAGGGG
CAGTGTCCCTTGTCCACGGCCAGCTCGTAGGCCGCGGCGGCCTTGCGGAGGCTGGTGTGCGTGAGG
GCGAAGGTGTCCCGGACCATGAACTTGACGTACTGGTGCTGGGGGTCCTCGGGGGCCATGACGCCC
TCCTCCCAGTCCGCGTAGTCGCGGCGCGGGCGGAAGGCGGGGTTGGGCAGGTTGAAGCTGATGTCA
TTGAAGAGGATGCGGCCGTTGCGCGGCATGAAGGTGCGGGTGACCAGGAAGGAGGGGGGCACCTCG
CGGCGGTGGGCGAGCACCTGCGCGGCCAGGACGATCTCATCGAAGCCCGAGATGTTGTGGCCCACG
ATGTAGACCTCCAGGAAGAGGGGCGGCCCGCGCAGGCGGCGGCGCCGCAGCTGGGCATAGGCCAGG
GGGTCCTCGGGGTCGTCCGGCAGGCCGGGGCCCCGCTCCTGCGCCAGCTCGGCGAGGTCTGGGTTG
TGGGCCAGCAGGTGCTGCCAGAGGGTGTCGGTGAGGCGGGCCTGCAGGGCGTGCCGCAGGGCCTTG
AAGGCGCGGCCGATGGCGCGCTTCTGCGGGCAGAGCATGTAGAAGGTGTGGGCTCGGGTCTCCAGC
GCTGCAGGCGGGCTCTGGACGGCCACCACCTGCAGCGCGGCGTCCAGCAGCTCCTCGTCCCCCGAG
AGGTGGAAGACCAGCAGGAAGGGCACGAGCTGCTTTCCGAAGCGGCCGTGCCAGGTGTAGGTCTCC
AGGTCATAGGTGAGGAAGAGGCGGCGGGTGCCCTCGGGGGAGCCGATGGGGCGGAAGGCGATGGTC
TGCCACCAGTCGGCCGTCTGGCGCTGAACGTGGTGGAAGTAGAAGTCCCGGCGGCGCACGGAGCAG
GTGTGGGCGGTCTGGAAGATGCGGCCGCAGTGCTCGCACTTCTGGGCCTCCTGGATGCTCTTGATG
AGGTGGCAGCGGCCCTGGGTGAAGAGCAGGCGGAGGGGGAAGGGGAGGCGGGGCGGCGGGCCCTCG
GGCGGGGGGTCCCAGCGCACGTGGTGCAGGTGGTGTTGCTGGCGGGTGACCACCTGGACGAAGGTG
GGCCCGGCGGCGCGGGCCAGCTCCACCGCGGTCTGGGGGGTAGCCTGCAGGAGGTCGGGGGGCGGG
CGCAGGAGGTGCAGCTGGAAGAGGTTGGCCAGGGCGCTGTCCCAGTGGCGGTGGTAGGTGATGCTC
CAGCTCTCCCCGTCCTGGGTGGTGCCCTGGAGGCGGAGGGTGGCGCGGCGCTCGAGCAGGAGCCCC
CGCGTGCCGGCCTCCGCGGCCTCGGCGGCGGCGGCCGGTCTCAGGCGGGCAGCTGGGCCAGGGGCA
CGGGCGCGTTGAGCTCGGGCAGCGGGAGGTGGTCGCGGCGCAGACGCGAGGCGTGGGCGATGACGC
GGCGGTTGATGTTCTGGATCTGCGGGTTCCCGGAGAAGACCACGGGCCCGGTGACTCGGAACCTGA
AAGAGAGTTCCACGGAATCAATGTCGGCATCGTGGGTGGCCACCTGGCGCAGGATCTCGGACACGT
CCCCGCTGTTTTCGCGGTAGGCGATGTCCTGCATGAACTGCTCGAGCTCGTCCTCGTCCAGGTCCC
CGTGGCCGGCGCGCTCCACGGTGGCGGCCAGGTCGACGGTGATGCGGTTCATGATGGCCACCAGGG
CGTTCTCTCCGTTCTCGTTCCACACGCGACTGTAGACCAGCTGGCCGTCGGCGTCCCGCGCGCGCA
TGACTACCTGGGCCAGGTTGAGCGCCACCAGGCGGTTGAAGGGCGCCTGCAGGCGCAGGGCGTGGT
GCAGGTAGTTGAGGGTGGTGGCGATGTGCTCGCAGAGGAAGAAGTTTATGACCCAGCGGCGCAGGG
TCAGCTCGTTGATGTCGCCCAGGTCCTCGAGGCGCTGCATGACCCGGTAGAACTCGGGGGCGAAGC
GAAAAAACTCGTGCTGGCGGGCCGAGACCGTGAGCTCCTCTTCCAGGGCGGCGATGGCCTCGGCCA
CCGCCTGCCGCACCTCCTCCTCTAAGGAGGGCGGGGGCGTGCTGGGTCCGGCCACCGCCGCCTCTT
CTTCCTCTTCTCCCTCCAGGGGTGGCATCTCCTCGTCTTCTTCTTCTGCTGCTGCTGCCTCCGCGG
GGACGGGGGGCGCAGGCCGGGGACGGCGCCGGCGCAAGGGCAGCCGGTCCACGAAGCGCTCGATGA
CCTCGCCCCGCATGCGGCGCATGGTCTCGGTGACGGCGCGGCCGCCCTCCCGGGGCCGCAGCTCGA
AGGCGCCCCCGCGCAGCGCGGTGCCGCTGCAGAGGGGCAGGCTGAGCGCACTGATGATGCAGCGTG
TCAACTCTCTCGTAGGTACCTCCTGCTGTTGCAGCGCTTCGGCAAACTCGCGCACCTGCTCTTCGG
ACCCGGCGAAGCGTTCGACGAAGGCGTCTAGCCAGCAACAGTCGCAAGGTAAGTTGAGCGCGGTGT
GCGTCGGGAGCCGGAGGTGCCGGCTGACGAGGAAGTGAAAGTAGGCCGTCTTGAGCTGCCGGATGG
CGCGCAGGAGGGTGAGGTCTTTGCGGCCGGCGCGCTGCAGGCGGATGCGGTCGGCCATGCCCCAGG
CCTCCTGCTGGCAGCGGCCGATGTCCTTGAGCTGCTCCTGCAGCAGATGTGCCACGGGCACGTCCC
GGTCGGCGTCCAGGTGGGTGCGACCGTAGCCCCGCAGGGGGCGCAGCAGCGCCAGGTCGGCCACCA
CGCGCTCGGCCAGGATGGCCTGCTGCATGCGCTGCAGGGAGTCTGAGAAGTCATCCAGGTCCAGGA
ACCGGTGGTAGGCGCCCGTGTTGATGGTGTAGGAGCAGTTGCCCAGCACGGACCAGTTGACCACCT
GGTAGTGGGGCTGGATGACCTCGGTGTAGCGCAGTCGACTGTAGGCGCGCGTGTCAAAGATGTAAT
CGTTGCAGAGGCGCAGCAGGTGCTGGTAGCCCACGAGCAGGTGGGGCGGAGGGTAGAGGTAGAGGG
GCCAGTGTTCCGTGGCCGGTTGGCGGGGGGAGAGGTTCATGAGCATGAGGCGGTGGTAGCGGTAGA
TGAAGCGGGACATCCAGGCGATGCCGACGGCGGAGACGGAGGCGCGGGTCCACTGGTGGGCGCGGT
TCCAAATGTTGCGCACCGGGCGGAAGAGCTCCACGGTGTAAATGGATTGCCCCGTGAGGCGGGCGC
AGTCGAGGGCGCTCTGTCAAAAAGAACCGGGTGTGGTTGGTTGGTGTGTGGTAGCGATCTATCTTT
CTTTGTGATCTTGGTAGTGAAGCCTGCCAGGCTCCAGCAGGGGGCGTCCGCCGTCTTTCCTTCCTT
CCCTATCTGGAGGTGTGTCTCTGTTCTCTTTTTTATTTCATGTAGCCATGCATCCCGTTCTGCGGC
AGATGAAGCCGCCGGCCGGCGCCCTGGGCGCGGAGGGGCGACGCGCTCTCGGTCGCCCTCGCCGT
CGCTGACGCGGCCGCGCGAGGAGGGGGAGGGCCTGGCGCGGCTGTCGGGCGCGGCGGCCCCCGAGC
GGCACCCACGGGTGCAGCTCAAGCGAGAGGCCATGGAGGCCTATGTGCCGAGGCAGAATGCGTTCC

FIG._1-3

GCGAGCGACCGGGGGAGGAGGGGGAGGAGATGAGGGACCTGCGGTTCCGCGCGGGGCGGGAGATGC
AGCTGGACCGGGAGCGAGTGCTCCAGCCCGAGGACTTTGAGGGGCGCGTGGAGGAGGCGGGGGGAG
TGAGCGCGGCGCGGGCCCACATGAGCGCGGCCAGCCTGGCCCAGGCCTACGAGCAGACGGTACGCG
AGGAGGTCAACTTCCAAAAGACCTTCAACAACAACGTGCGCACCCTGGTGAGCCGGGACGAGGTGA
CCATGGGACTGATGCACCTGTGGGACTTTGTGGAGGCCTTCCTGCAGCACCCCCGGTCCCGCGCGC
TGACCGCGCAGCTGCTGCTGATCGCGCAGCACTGCCGGGACGAGGGCATGGTGAAGGAGGCGCTGC
TGAGCCTGGGCGCGCCCGAGAGCCGCTGGCTGGTGGACCTGGTGAACCTGCTCCAGACCATTGTGG
TGCAGGAGCGGTCCATGAGCCTGAGCGAGAAGGTGGCGGCCATCAACTACTCGGTGGCGACCCTGG
CCAAGCACTACGCGCGCAAGATCTCCACCTTCTACATGCGCGCGGTGGTGAAGCTGCTGGTGCTGG
CCGACAACCTGGGCATGTACCGCAACAAGCGGCTGGAGCGCGTGGTCAGCACCTCGCGGCGGCGCG
AGCTCAATGACAAGGAAGCTCATGTTTGGCCTCCGCCGGGCGCTGGCCGGGGAGGGCGAGGAGGAC
CTGGAGGAGGAGGAGGACCTGGAGGAGGCGGAGGAGGAGGAGCTGGAAAGAGGAGGAGTTCGGTCC
CCGGGGACCGCGGCGCGTGAGGTGGCAGTCCCCGCTGACTGCGAGCGATGAGGGTGATGTGTACTG
ATGGCAACCATCCCCCTTTTTAACAACAACAGCAGCATGGCGGCGAGCTCTGAAGCTGGGGCGGCG
GCGGCGGGGGTGAGCGCGGCCTCCCTGGCGCCCGAGCGGGCGACGCGGATGCAGGCGCTGCCCTCC
CTGGACGAGCCTTGGGAGCAGGCTCTGCGGCGCATCATGGCGCTGACGGCCGACGGGTCTCGGCGC
TTCGCGAGCCAGCCCCTGGCCAACCGCATCGGGGCCATCCTGGAGGCGGTGGTGCCTCCGCGCACG
AACCCGACGCACGAGAAGGTGCTGACCGTGGTGAACGCGCTGCTGGAGACCTCGGCCATCCGCCCG
GACGAGGCCGGCATGGTGTACGATGCGCTGCTGGAGCGGGTCTCCCGCTACAACAGCGGCAACGTG
CAGACCAACCTGGACCGGCTGTCCCAGGACGTGCGGCAGGTGATCGCCCAGCGCGAGCGCTCGAGC
GCCAACAACCTGGGCAGCCTGGCCGCGCTGAATGCCTTCATCGCCTCGCTGCCCGCAACGGTGGAG
CGGGGCCAGGAGAGCTACCTGGGGTTCCTCAGCGCGCTGCGGCTGCTGGTGAGCGAGGTGCCGCAG
ACGGAGGTGTTCCGCTCGGGGCCGCACACCTTCCTGCAGGCGGCGCGGAACGGTTCCAAGACGGTG
AACCTCAACCAGGCCATGGAGAACCTGCGGCCCCTGTGGGGGCTGCAGGCCCCCGCTGGGGAGCGC
GGGCACGTGTCCTCCCTGCTGACGCCCAACACCCGGCTGCTGCTGCTCCTGGTGGCTCCCTTCGCG
GAGGAGATGAACGTCAGCCGGAGCTCCTACATTGGGCACCTGCTGACACTCTACCGCGAGACGCTG
GCCAACTTGCATGTGGACGAGCGCACGTACCAGGAGATCACCAGCGTCAGCCGGGCGTTGGGCGAC
GAGGACGACGCGGCGCGGCTGCAGGCCACCCTCAACTTCTTCCTGACCAACCGGCAGCGGCGGCTG
CCGGCGGCGTATGCCCTGACCGCCGAGGAGGAGCGCATCCTGCGCTACGTGCAGCAGGCCGTGAGC
CTGTACCTGATGCAGGACGGGGCGACGGCCACGGGCGCCCTGGACGAGGCCAGCCGCAACCTGGAG
CCCAGCTTCTACGCGGCGCACCGGGACTTCATCAACCGCCTGATGGACTACTTCCATCGCGCGGCC
GCGGTGGCGCCCAACTACTTTATGAATGCCGTCCTGAACCCCCGCTGGCTGCCCTCGGAGGGCTTC
TTCACCGGCGTGTATGACTTCCCGGAGCAGGACGAGGGGGAGGAGCGGCCCTGGGACGCCTTTGAC
AGCGACGAGGAGGGCCGCCTCATGCTGCGGTCCGCAGCCTCCTCAGAGCCCTCCTCCTCCTTCACC
CCCCTGCCCCTGACCGAGGAGCCGCCCTCGCGGCCCTCCACCCCGGCCCTCTCGCGCGTCCCGTCC
CGGGCATCCTCCCTGCTCTCTCTGGCCTCTCTGGGAAAGCGGGAGGGAGGGGACTCGCTCGCCTAC
TCGCCGGCCACGCCCACCTATGGCTCTCGCTGGGGCTCGCGCCGCTCCAGCCTGGCCAGCGGCGCC
GACAGCCTGGAGTGGGACGCGCTGCTGGCCCCTCCCAAGGATGTGAACGAGCACCCAGGCGCCGCC
GCCGGCCGCCGCCGCCGCGCCTCCCGCTCCTCCCTGGAGGAGGACATCGACGCCATCAGCAGCCGG
CTGTTCACCTGGCGCACGCGCGCCCAGGAGATGGGCCTGCCCGTGGCCAGCTTCTCCCGCCGCCAC
CAGCCGCGCCCCGGGGCCCTCGAAGACGACGAGGAGGAGGAAGACTGGCGCCAGGACCGGTTCTTT
CGCTTCGAAGCGCCCGAGGAAAACCCCTTCCGCCACATCGCCCCCAAGGGGCTGTAATGCAAAAAA
GCAAAATAAAAAACCCCTCCCGGTCCAACTCACCACGGCCATGGTTGTCCTTGTGTGCCCGTCAGA
TGAGGAGGATGATGCCAGCAGCGCCGCCGCAGGGAGCGTCGCCTCCGCCGTCCTACGAGAGTGTGG
TGGGGTCTTCGCTCACGGAGCCTCTTTATGTGCCGCCGCGGTACCTGGGCCCCACCGAGGGGCGGA
ACAGCATCCGTTATTCACAGCTCCCGCCGCTCTACGATACCACAAAGATCTATCTGATCGATAACA
AGTCGGCGGATATCGCCAGTCTGAACTACCAAAACAACCACAGTGACTTTCTCACCAGCGTGGTGC
AGAACAGCGACTTCACGCCCATGGAGGCGAGCACGCAGACCATCAACCTGGATGAGCGCTCGCGCT
GGGGCGGGGAGTTTAAGAGCATTCTGACCACCAACATCCCCAACGTGACCCAGTACATGTTCAGCA
ACAGCTTCCGGGTGCGCCTGATGAGCGCGCGCGATAAAGAGACAAATGCCCCCACCTACGAGTGGT
TCACCCTGACCCTGCCCGAGGGCAACTTCTCGGACATCGCGGTCATCGACCTGATGAACAACGCGA
TCGTGGAGAACTACCTGGCGGTGGGGCGGCAGCAGGGGGTCAAGGAGGAGGACATCGGGGTGAAGA
TCGACACGCGCAACTTCCGCCTGGGCTATGACCCGGAGACCAAGCTGGTCATGCCCGGCAGCTACA
CCAACATGGCCTTTCACCCCGACGTGGTGCTGGCACCGGGCTGCGCCATCGACTTCACCTTCTCCC
GCCTAAACAACCTGCTGGGCATCCGCAAGCGCTACCCCTACCAGGAGGGCTTCATGCTGACCTACG
AGGACCTGGCGGGGGGCAACATCCCCGCGCTGCTGGACCTCACCACCTATGATCAGGAGAACTCCA

FIG._1-4

GCACCATCAAGCCCCTGAAGCAGGACAGCAAGGGTCGCAGCTACCACGTGGGCGAGGACCCCGAGG
CGGGGGACACCTTCACCTACTACCGCAGCTGGTACCTGGCCTACAACTACGGGGACCCGGCCACGG
GCACCGCCTCCCAGACGCTGCTGGTCTCCCCGGACGTAACCTGCGGAGTGGAGCAGGTCTACTGGA
GCCTGCCGGACCTGATGCAGGACCCGGTGACCTTCCGGCCCAGCCAGACGCCGAGCAACTACCCGG
TGGTAGCCACGGAGCTACTGCCGCTGCGCTCCCGGGCCTTCTACAACACCCAGGCCGTGTACTCCC
AGCTCCTGCAGCAGGCCACCAACAACACCCTGGTCTTTAACCGCTTCCCGGAGAACCAGATCCTCC
TGCGCCCGCCAGAGTCCACCATCACCTCCATCAGCGAGAACGTGCCCTCGCTGACGGACCACGGCA
CGCTGCCGCTGCGTAACAGCATCCCCGGGGTGCAGCGGGTAACCGTCACCGACGCGCGGCGCCGCG
TGTGTCCCTATGTGTACAAGAGTCTCGGGGTGGTGACCCCGAGGGTGCTCAGCAGCCGAACCTTCT
AACCGACAGCCCTACCCGTCACAGGGGAGACAGAGAAAAGACAGCCAGCCCCGCCATGGCCATCCT
CGTCTCGCCCAGCAACAACTTTGGCTGGGGACTGGGCCTGCGCTCCATGTACGGGGGCGCCCGCCG
CCTGTCCCCGGATCACCCCGTGATCGTCCGACGCCACTACCGGGCCAACTGGGCCAGTCTGAAGGG
ACGCGTGGCCCCCAGCACCATAGCGACAACGGATGACCCTGTGGCCGACGTGGTCAACGCGATCGC
CGGCGCCACCCGCCGCCGGCGCCGCCATCGTCGACGTCGGAGGGCCGCGCGCGTCTCCTCCGTGGC
CGTCACCGGGGACCCGGTGGCCGATGTGGTCAACGCGGTGGAGGCGGTAGCCCGGCGCCGCCGCGC
GCGGCGCCGTTCTTCGCGCATGCAGACCACGGGGGACCCCGTGGCGGATGTGGTGGCGGCGGTGGA
AGCGGTGGCGCGCCGGAGGCGGAGCACCCGGCGGCGGCGCAGGCGCTCCGCGCCGGCCATCCTGGG
GGTGCGCCGCAGCCGCCGCCTCCGCAAACGCACCTCGTCCTGAGATTTTTGTGTTTTGTTTTTTCT
GCCTCCCGTGGGTGAACAAGTCCATCCATCCATCCAACATCCGTGGCTGCTGTGTCTTTGTCTTTT
CTTTGCGTTGCGCCCCAGTTGAGCCGGCACCGACGCGCTCGGCCATGGCCATCTCGCGCCGCGTGA
AAAAGGAGCTGCTGCAGGCGTTGGCGCCCGAGGTGTACGGGGCGCCTAAGAAGGAGGAGAAGGACG
TCAAAGAGGAGTCCAAAGCTGACCTTAAACCGCTGAAGAAGCGGCGCAAGGCCAAGCGGGGGTTGA
GCGACAGCGACGAGGTGCTGGTGCTGGGCACGCGCCCCAGGCGCCGCTGGACGGGGCGGCGCGTGC
GCGCCCACCTACCGCCCGGTGCCAGCCTCGCCTACGTCCCGGGTCTTCGGAGGTCGAGCGCCACCA
AGCGCTCTGCGGACGAGTTGTATGCGGACACGGACATCCTGCAGCAGGCGTCCCAGCGCCTGAACG
AATTTGCTTATGGCAAGAGAGCCCGGCGGCAGCGGCGGGCCCGCCCCTCGCCGACCCCCGCGTCCC
GCGGCCGGACCACCAAGCGCTCTTATGACGAGGTCGTGGCAGACAGTGACATCCTGCAGCAACTTG
GATCCGGGGACCGCTCCAATGAGTTCTCCTATGGCAAGCGGTCGCTGCTGGGGGAGTCAGGAGACA
CCGTCCCGGCTGTGGCCGTCCCGCTGGAGGAAGGCAGGAACCACACACCCAGCCTGCAGCCGCTCA
CCGAGCCCATGCCCCTGGTGTCCCCTCGCACGGCCGTCAAGCGCCGGGCGCCCGCCGACGAGCCCA
CCGCCTCACTGGTCCCCACCGTGCAGGTCCTGGCCCCCAAGCGTCGTCTGCAGGAGGTGGTGGTGG
AGCCGCCCGCTCCAGCACCCACGCCGCCCCTAGCCCCGCGGCGGTCCAGCCGGCGCATCATTCTGG
CTCCGCGCCGGGCGGGCCGGCCCCAGGCCGTCGTGGCGCCGCAGCTCAGCGCGGCCGCGGCGCTGG
AGCGGGCGGCGGCCGCCGTGCCCCTGCCACCGGACACGGAGGACGACCTGGTGGAGATGGCAGAGG
CTGTCGCCGCGCCCGAGGTGCTGCCCAGCCTCCCCGTCTCCATCATGCCGCCCACCGCCACGGAGG
TGGCCCTGCCCGTACAGACCCCACTGCCGCCCGTGGCGGTGGCCAAGAGCTCCCTGACCCCCGGCC
TCCGCGCGCTGATGGGCACCGAGCGGGTGCCGGTTCCAGTCCTGGAGGCGCCCCTGGTGGCCATGC
CCGTGCTCCGGGCCACCACCGCCCGTGCCGAGCCCCCGCGCCGCGTGCCCCGCAGGGCCGTGCGGG
ACATCCCGGCCAGGCAGCCCCGCACGGTATCCCTGCCCGTGCTCACGGAGCCCGGCCCGGCCACCG
CGGTCGCCTCCGTGCGCGCGGCAGCCCAAGTCCTGCAGGCGCCCCCCGCCCGACCGGCCACCGTCT
CCGTGGGGGTGGGCACCGAGCCGGTGGTGCAGTCCATCACGGTCAAGCGGTCAAAGCGCCTGACCA
AGCACCATCGGGGTGCAGACCATCGACGTCACCGTGCCCACCGTCCGCACTGTCAGCGTGGGCACC
AACACGCCCCGGCTGAGGAGCGCCTCGGTGGGCGTCCAGACCGCTCCCGAGACCCGCTCCCAGGGG
GTGCAGGTGGCTTTCCAACCAGCGTGCTAGCCCACCGCACACCCAGGCAGGTGCGGCTGACGGCGG
TGGTGCCCCCCACCCCGCGCGCCCCGGTGGTTCCGGTGGCCCGGCGCCCGCGGCGGTTCCGGTGCC
TCCCCCAGCCCCTCCAGCCCCGCGCGCGCCGCGTGCGCCTCGCGCCCCCAGAGCGCCTCGGCGTCG
CCGCCGTACCCCGGTGGCGGTGGCAGCGCCGCCCGCCCGCAGCGGCGGTCCCCCGCCCTCGGCTGC
CGAGGCGGCCCATCGTGCTGCCCGGGGTGCGCTATCATCCCAGTCAGGCCATGGCTCCCACCGCCC
AACGCGTCATCTGGCGTTGATTTATTTTTGGAGACCTGACTGTGTTGTGTTCCTTAAATTTTTTAT
CCTCCTCCTCCTCTGCTGAAGCCAGACGATGCTGACCTACCGGTTGCGGCTGCCCGTGCGGATGCG
GAGACCGAGACTCCGCGGTGGGTTCCGCGTGGCGCCTCGGCGCAGCGGCGGCAGGCGGCGGTACCG
CCGGGGGCCGATGAGGGGTGGCATCCTGCCGGCGCTGGTGCCCATCATCGCGGCATCCATCTGGGC
CATCCCCGGCATCGCCTCGGTGGCGATGAGTGCTAGACAACGCAATTAACGGCGCTGCTGTGTATG
TGTGTCTTCCATGTGCCTTCCTTCCTTCGTTCCCAACGGAACAGCAGCACCGTCTCCATGGAGGAC
CTAAGCTTTTCCGCGTTGGCTCCACGCTTTGGCACGCGGCCGGTCATGGGCACTTGGAGCGAAATC
GGCACGAGTCAGATGAACGGCGGCGCGCTCAGCTGGAGCAATATCTGGAGCGGGCTGAAGAGCTTT

FIG._1-5

GGTAGTTCTCTGGCCTCCACGGCCAACAAGGCCTGGAACAGCGGGACGGTGACGAGCGTGCGCAAC
AAGTTGAAGGATGCCGACGTGCAGGGGAAGATAGGTGAGGTCATTGCCTCCGGGGTCCACGGTGCC
CTGGACGTGGCCAACCAGGCCGTCTCCCACGCCGTGGACCGCCGGTGCAACAGCAGCAGCTGCGGC
AGCAGCAGCTCCTCCGCCAGCAGCAGCAACAGATGGGCCTCGTGGAACCCTCCTATGAGATGGAGA
CAGACGAGCTGCCTCCTCCCCCCGAGGACCTCTTGCCTCCTCCTCCTCCTCCGCCGCCTGCCTCGG
CCACTCCCGCGCGCCAATCCCGCGGGACGTCCCGCCAAGCGCCCGCCGCCGCCCAGGAGATCATCA
TCCGCTCCGACGAGCCCCCTCCCTATGAAGAGCTGTATCCCGACAAGGCCGGGATCCCCGCCACCT
TGGAGCTGCGTCCCGAGACCAAACTGCCCGCCGTGGCCCACAATAAGATGCGCCCCCCGCCGCCGC
TCACCACCACCACCTCCTCCGCTGCCGCCGCCGCCCCCGCCCCGGCCCCCGCGGCTCCTGTGCGTC
GGCGTCCGGCCGCGGCTCCGGCCGCGGCTCCGGCGAGTTCCAAAGGCCCCCCAGGTGGGGGTCCGC
GCGCGCGGGTGGCAAAACAAACTCAACACCATTGTGGGACTGGGTGTCCGCACATGCAAGCGCCGT
CGTTGTTACTGAGAGAGACAGCATGGAGAAACAACAATGTCTGGATTCAAATAAAGACACGCCTAT
TCTTCCACGGTGCTCCGCGCTGTGTTATTTTCAACGGGCTGTTTCCTTTTGCATCTCTGTGCCATC
GCGCCACGGGGAATTCCGCAGGATGGCGACGCCGTCGATGATGCCGCAGTGGTCCTATATGCACAT
CTCCGGGCAGGACGCGTCCGAGTACCTGTCTCCCGGGCTGGTGCAGTTCTCCCAGGCGACGGAGAC
CTACTTTAACCTGAACAACAAGTTTAGGAACCCCACCGTCGCGCCCACCCACGATGTGACGACGGA
GCGCTCGCAGCGGCTGCAGCTGCGCTTCGTCCCCGTGGACAAGGAGGACACTCAGTACACATACAA
GACCCGCTTCCAGCTGGCGGTGGGCGACAACCGCGTGTTGGACATGGCGAGCACCTTCTTTGACAT
CCGGGGAACGCTGGACCGGGGACCCTCCTTCAAACCGTACTCGGGCACCGCGTACAACATCATGGC
TCCCAAGAGCGCTCCCAACAACTGTCAATATCTAGACCCTAAAGGTGAAACTGAGGCTGGCAAAGT
TAATACCATTGCTCAAGCAAGTTTTGTGGGTCCTATTGATGAAACCACGGGAGACATTAAAATTAC
AGAAGAAGAAGACGAAGAGACCACCATCGATCCTTTGTATGAGCCCCAACCCCAGCTTGGTCCAAG
CTCGTGGTCAGACAATATACCTTCTGCGACTAGCGGAGCTGGAAGAGTTCTCAAACAGACCACACC
GCGTCAACCTTGTTACGGTTCTTATGCCTCTCCGACAAATATTCACGGTGGGCAAACGAAGGATGA
CAAGGTTACACCATTGTACTTTACAAACAATCCCGCCACCGAAGCCGAAGCACTCGAAGAAAATGG
ATTAAAGCCAAATGTCACCCTATACTCAGAGGATGTTGACCTAAAAGCACCAGATACTCATCTGGT
CTATGCTGTGAATCAAACCCAGGAATTCGCTCAATATGGACTTGGACAACAGGCCGCTCCAAACAG
GGCCAATTACATCGGCTTCAGGGACAACTTTATCGGGCTGTTGTACTACAACAGCAATGGCAACCA
GGGCATGCTAGCCGGTCAGGCCTCTCAGCTCAACGCGGTGGTCGACCTGCAGGACAGGAATCACCG
AACTAGCTACCAGCTCTTCCTCGATAGCCTCTATGACAGGTCGAGGTACTTTAGCCTGTGGAACCA
GGCCATCGATTCTTATGACAAGGATGTGCGTGTGCTGGAAAACAATGGCGTGGAGGACGAGATGCC
CAACTTTTGCTTTCCCATCGGCGCCATCGAGACCAACATGACATTTACACAGCTCAAAAAGAGTGA
GAATGGTGGCTCAAGAGCCACAACCTGGACAAAGGAGAATGGGGATGATGGCGGAAACGGAGCGGA
GCACTACCTGGGCATCGGCAACCTCAACGCCATGGAGATCAATCTCACGGCCAACCTCTGGCGCAG
CTTCCTCTACAGCAACGTGGCGCTGTACCTGCCTGACAAGTACAAGTTTTCCCCGCCCAACGTCCC
CATCGACCCCAACACGCACTCCTATGACTACATCAACAAGCGCCTGCCCCTCAACAACCTCATTGA
TACCTTTGTCAACATCGGGGCGCGCTGGTCCCCGGATGTCATGGACAACGTCAACCCCTTCAACCA
CCACCGCAACTACGGCCTGCGCTACCGCTCCCAGCTCCTGGGCAACGGCCGCTACTGCAAGTTCCA
CATCCAGGTGCCGCAAAAGTTCTTTGCCCTCAAGAGCCTGCTGCTCCTGCCGGGGGCGACCTACAC
CTACGAGTGGTCCTTCCGCAAGGACGTCAACATGATCCTCCAGTCCACGCTGGGCAACGACCTCCG
CGCGGACGGGGCCAAAATCAACATCGAGAGCGTCAACCTCTACGCCAGCTTCTTTCCCATGGCCCA
CAACACCGCCTCCACCCTGGAGGCCATGCTGCGCAACGACACCAACAACCAAACCTTTATTGACTT
CCTCTCCTCCGCCAACATGCTCTACCCCATCCCGGCCAACGTCACCAACCTGCCCATCTCCATTCC
CAGCCGCAACTGGGCCGCCTTCCGCGGCTGGAGCTTCACGCGGCTGAAGCACAACGAGACCCCCGC
CCTGGGCTCGCCCTTCGACCCCTACTTTACCTACTCGGGCTCCATCCCCTACCTGGACGGGACCTT
CTACCTGGGCCACACCTTCCGCCGCATCAGCATCCAGTTCGACTCCTCCGTGGCCTGGCCGGGCAA
TGACCGCCTGCTCACTCCCAACGAGTTCGAGGTCAAGCGCACCGTGGACGGGGAGGGCTACACGGT
GGCCCAGACCAACATGACCAAAGACTGGTTCCTGGTGCAGATGCTCGCCCACTACAACATCGGCTA
CCAGGGATACCACCTGCCAGAGGGCTACCGCGACCGCACCTACTCCTTCCTGCGCAACTTTGAGCC
CATGTGCCGCCAGGTGCCCGACTACGCCAACCACAAAGATGAGTACCTGGAGGTGCCCACCACCAA
CCAGTTCAACAGCAGCGGCTTTGTATCCGCGGCCTTCACCGCCGGCATGCGCGAGGGGCACCCATA
CCCCGCCAACTGGCCCTACCCGCTCATCGGCGAAGACGCCGTGCAGACCGTGACCCAGCGCAAGTT
CCTCTGCGACCGCACGCTCTGGCGCATCCCCTTCTCCTCCAACTTCATGTCCATGGGCACCCTCAC
CGACCTGGGCCAGAACCTCCTCTACGCCAACTCGGCCCACGCCCTCGACATGACCTTCGAGGTCGA
CGCCATGGATGAACCCACCCTCTTGTATGTTCTGTTCGAGGTCTTTGACGTCTGCGGCGTGCACCA
GCCGCACCGAGGCGTCATCGAGGCCGTCTACCTGCGCACGCCCTTCTCCGCCGGGAACGCCACCAC

FIG._1-6

CTAAGGCGGAGCCGCGCAGGCATGGGCAGCACCGAGGACGAGCTCCGAGCCATGGCGCGCGACCTC
CAGCTGCCCCGCTTCCTGGGCACCTTTGACAAGTCCTTCCCGGGCTTCTTGCAAGAGTCCCAGCGC
TGCTGCGCCATCGTCAACACGGCCGCCCGCCACACCGGAGGCCGCCACTGGCTGGCCGTCGCCTGG
GAGCCCGCCTCGCGCACCTTCTACTTCTTTGACCCCTTCGGCTTCTCCGACCGGGAGCTCGCCCAG
GTCTATGACTTTGAGTACCAGCGCCTGCTGCGCAAGAGCGCCATCCAGAGCACCCCGGACCGCTGC
CTCACGCTCGTCAAGAGCACCCAGAGCGTGCAGGGACCGCACAGCGCCGCCTGCGGACTCTTCTGC
CTCCTCTTCCTCGCCGCCTTTGCCCGCTACCCCGACAGCCCCATGGCCTACAATCCCGTCATGGAC
CTGGTGGAGGGCGTGGACAACGAGCGGCTCTTCGACGCCGACGTCCAGCCCATCTTCCGCGCCAAC
CAGGAGGCCTGCTACGCGTTCCTCGCTCGCCACTCCGCCTACTTCCGCGCCCACCGCCACGCCATC
ATGGAACAGACACACCTGCACAAAGCGCTCGATATGCAATAAAGGCTTTTTATTGTAAGTCAAAAA
GGCCTCTTTTATCCTCCGTCGCCTGGGGGTGTATGTAGATGGGGGGACTAGGTGAACCCGGACCCG
CCGTCGGCTCCCCTCCATCCCCTCTTCTCTCAAAACAGGCTCTCATCGTCGTCCTCCGTTCCCACG
GGGAAGATGGTGTTCTGCACCTGGAACTGGGGCCCCCACTTGAACTCGGGCACCGTCAGTGGAGGC
CGCGTCTGCATCAGGGCGGCCCACATCTGTTTGGTCAGCTGCAGGGCCAGCATCACATCGGGGGCG
CTGATCTTGAAATCACAATTCTTCTGGGGGTTGCCGCGCGACCCGCGGTACACCGGGTTGTAGCAC
TGGAACACCAGCACCGCGGGGTGGGTCACGCTGGCCAGAATCTTGGGGTCTTCCACCAGCTGGGGG
TTCAGCGCCGCCGACCCGCTCAGCGCGAAGGGGGTGATCTTGCAGGTCTGCCGGCCCAGCAGGGGC
ACCTGGCGGCAGCCCCAGCCGCAGTCGCACACCAGCGGCATCAGCAGGTGCGTCTCCGCGTTGCCC
ATCCGGGGGTAGCAGGCCTTCTGGAAAGCCTTGAGCTGCTCGAAGGCCTGCTGCGCCTTGGAGCCC
TCCGAGTAGAAGAGGCCGCAGGACCGCGCCGAGAAGGTGTTGGGGGCCGACCCCACGTCGTGGCTG
CAACACATGGCCCCGTCGTTGCGCAGCTGCACCACGTTGCGGCCCCAGCGGTTGGTGGTGATCTTG
GCGCGCTCGGGGGTCTCGCGCAGGGCGCGCTGCCCGTTCTCGCTGTTGAGATCCATCTCCACCAGC
TGCTCCTTGTTGATCATGGGCAGCCCGTGCAGGCAGTGCAGCCCCTCCGAGCCGCTGCGGTGCTGC
CAGATCACGCACCCGCAGGGGTTCCACTCGGGCGTCTTCAGACCCGCCGCCTTCACCACAAAGTCC
AGCAGGAAGCGGGCCATCACTGTCAGCAGGCTCTTTTGCGTGCTGAAGGTCAGCTGGCAGCTGATC
TTGCGCTCGTTCAGCCAGGCTTGGGCCCCGCGCCGGAAGCACTCCAGGGTGCTGCCGTCCGGCAGC
AGCGTCAGGCCCTTGACATCCACCTTCAGGGGGACCAGCATCTGCACAGCCAGATCCATGGCCCGC
TGCCACTTCTGCTCCTGAGCATCCAGCTGCAGCAGCGGCCGGGCCACCGCCGGGCTCGGGGTCACC
GGGCGCGGGGGGCGGGCCCCCTCCTCTTCCTCCCCATCTTCGCCCTTCCTCCTCGCGGGCCGCGCC
GTCGCCGCTGCCGTCTCTTCAGCCTCGTCCTCCTCCTCCTCGCTGACCAGGGGCTTGGCACGCGCG
CGCTTCCGCCGCTCCTGCACGGGCGGAGAGGCCGCGCGCTTGCGGCCTCCCCCGCGCCGGCTGGGG
GTCGCGACAGGAGCGTCGTCCACAATCAGCACCCCCTCTTCCCCGCTGTCATAGTCAGACACGTCC
GAATAGCGGCGACTCATTTTGCTTCCCCTAGATGGAAGACCAGCACAGCGCAGCCAGTGAGCTGGG
GTCCTCCGCGGCCCCGACCCTTCCGCCGCCACCACCGCCGCCACCTCCGCCCACGTCACCGCCACC
TTCACTGCAGCAGCGGCAGCAGGAGCCCACCGAAACCGATGACGCGGAGGACACCTGCTCCTCGTC
CTCCTCGTCCTCCGCCTCCAGCGAGTGCTTCGTCTCGCCGCTGGAAGACACGAGCTCCGAGGACTC
GGCGGACACGGTGCTCCCCTCCGAGCCCCGCCGGGACGAGGAGGAGCAGGAGGAGGACTCGCCCGA
CCGCTACATGGACGCGGACGTGCTGCAGCGCCACCTGCTGCGCCAGAGTACCATCCTGCGCCAGGT
CCTGCAGGAGGCCGCCCCCGGCGCAGCCGCGGAGGCCGCCGAGGCGCCCTCGGTGGCGGAGCTCAG
CCGCCGCCTGGAAGCGGCCCTCTTCTCCCCCGCCACGCCGCCGCGGCGCCAGGAGAACGGAACCTG
CGCCCCGGACCCCCGCCTCAACTTCTACCCGGTCTTCATGCTGCCCGAGGCCCTGGCCACCTACCT
CCTCTTCTTCCACAACCAAAAGATCCCCGTCAGCTGCCGCGCCAACCGCCCACGAGCCGACGCGCA
CTGGCGGCTGCCCAGTGGGACCCCCTTACCTGACTATCCAACCACCGACGAGGTTTACAAGATCTT
TGAGGGCCTGGGGGACGAGGAGCCGGCCTGCGCCAACCAGGACCTGAAAGAGCGCGACAGCGTGTT
AGTCGAGCTCAAGCTGGACAACCCCCGCCTGGCGGTGGTCAAGCAGTGCATCGCCGTCACCCACTT
CGCCTACCCGGCCCTGGCGCTGCCACCCAAGGTCATGAGCACGCTCATGCAGACCCTGCTGGTGCG
CCGCGCGAGCCCACTCCCCGACGAGGGCGAGACGCCCCTCGAGGACCTCCTGGTGGTCAGCGACGA
GCAGCTGGCCCGCTGGATGCACACCTCGGACCCCAAGGTCCTGGAGGAGCGGCGCAAGACCGTCAC
CGCCGCCTGCATGGTCACGGTGCAGCTCCACTGCATGCACACCTTCCTCACCTCCCGCGAGATGGT
GCGCCGCCTCGGAGAGTGCCTCCACTACATGTTCCGCCAGGGCTACGTCAAGCTAGCTAGCAAGAT
CGCCAATATGGAACTCTCTAACCTGGTCTCCTACTTGGGCATGCTGCACGAAAACAGGCTCGGTCA
GCACGTGCTCCACCACACCCTCAAGCATGAGGCGAGACGCGACTACGTCCGGGACACCATTTACCT
ATACCTGGTCTATACCTGGCAGACCGCCATGGGGGTCTGGCAGCAGTGCCTCGAGGACCGAAACCT
GCGCGCCCTGGAAACGTCTCTGGCTCGCGCTCGCCAGAGCCTGTGGACGGGCTTTGATGAGCGCAC
TATCGCGCAGGACCTCGCCGCGTTCCTTTTCCCCACCAAGCTCGTAGAGACCCTGCAGCGCTCGCT
CCCCGACTTTGCCAGCCAGAGCATGATGCATGCCTTCCGCTCCTTCGTCCTCGAGCGCTCCGGCAT

FIG._1-7

CCTGCCCGCCGTCTGCAACGCGCTCCCCTCTGACTTTGTGCCCACCGTCTACCGCGAGTGCCCGCC
GCCCCTCTGGGCTCACTGCTACCTCCTGCGCCTCGCCAACTTCCTCATGTACCACTGCGACCTCGC
CGAGGACACCTCCGGCGAGGGCCTCTTTGAGTGCTACTGCCGCTGCAACCTCTGCGCACCGCACCG
CTGCCTCGCCACCAACACCGCCCTCCTCAACGAGGTGCAAGCCATCAACACCTTTGAGCTCCAGCG
GCCCCCCAAGCCCGACGGCACCCTGCCACCGCCCTTCAAGCTGACCCCCGGTCTCTGGACCTCCGC
CTTCCTCCGCCACTTTGTCTCCGAGGACTACCACTCGGACCGCATCCTCTTCTACGAGGACGTGTC
CCGCCCCCCCAGGGTGGAGCCCTCCGCCTGCGTCATCACGCACTCGGCCATTCTCGCGCAATTGCA
TGACATCAAAAAGGCCAGGGAAGAGTTTTTGCTGACCAAAGGCCACGGCGTCTACCTAGACCCCCA
CACCGGAGAGGAGCTCAACACCGCCGCCCCGTCCACCGCCCACCATGCCGCCCCTCCGGAGGAAGC
CCATCCGCAGCAGCACCAGCACCAGCAGCAGCCGAGCCACCGCCGCCGCCACCACCGCTCCAGCTA
CGCAGACCGTGTCCGAAGCGAGCTCCACGCCTACGGCGGTGCGACCGGTTCCTCCCGCGACCCTGT
CTCTGGCGGATGCTCTGCCAGAGGAACCCACTCCCGCGATGCTGCTCGAAGAAGAGGCTCTCAGCA
GCGAGACCAGCGGCAGCTCCGAAGGCAGTTTGCTCAGTACCCTCGAGGAACTGGAGGAGGAGGAGG
AACCGGTCACACCGACGAGGCCATCCAAGCCCTCCTACACCAACAGCAGCAGCAGCAAGAGCATCA
GCCAGCGCAGGAACTCCGTCGTCCCCAGCGAGGCTCGTAGATGGAATCAGACATCCATCCACCGGA
GTAGCCAGCCAGGTAGGACACCTCCGCCCTCGGCCCGCCGACGCTCCTGGCGCCGCTACCGCCACG
ACATCCTCTCGGCCCTGGAGTACTGCGCCGGAGACGGAGCCTGCGTGCGCCGGTACCTACTCTACC
ACCACAACATCAACATCCCTTCCAAGATCATCCGTTACTACAAATCCTCTTCCCGTTCCAGCGATC
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TTCCCCACGCTGTACGCCATCTTCCAGCAGAGCCGCGGCGGCCAGGACGCCCTCAAAATCAGGAAC
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FIG._1-8

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FIG._1-9

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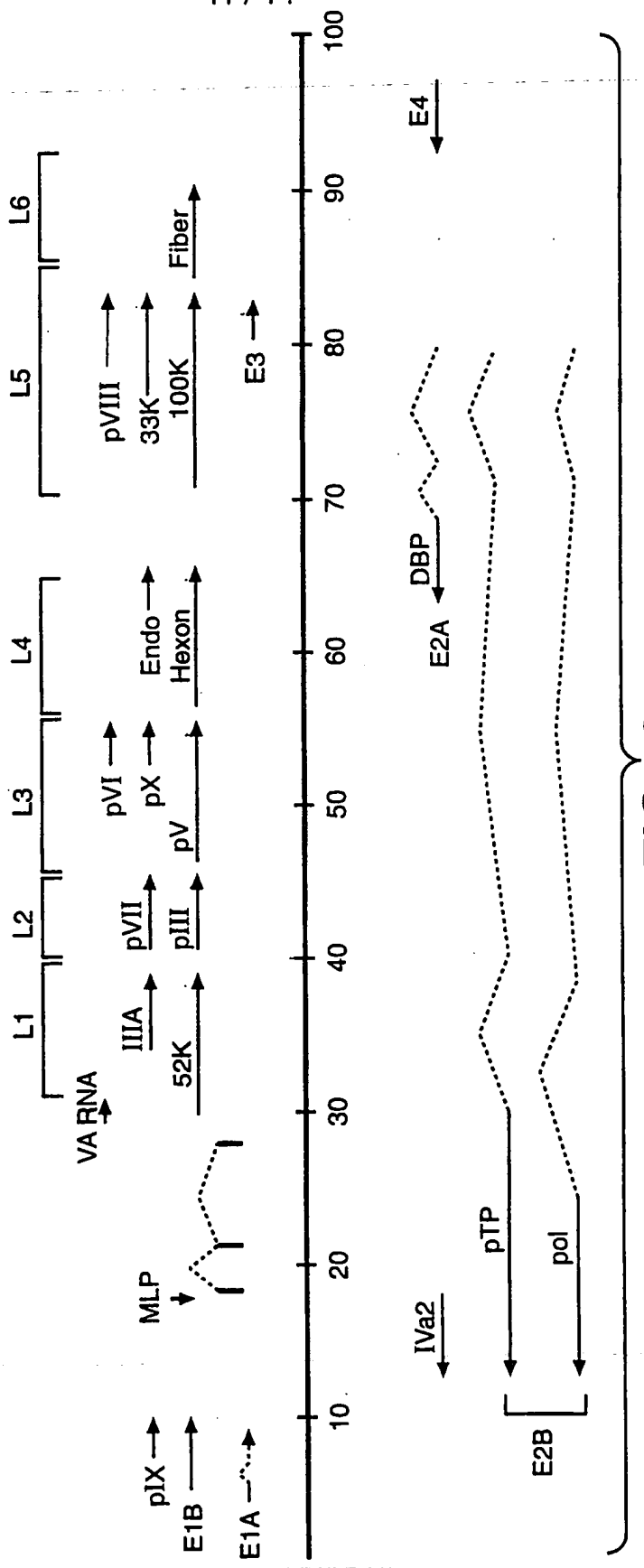
FIG._1-10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pV
pIII
pVII
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IVa2
pTP
pol
E2B

FIG._2

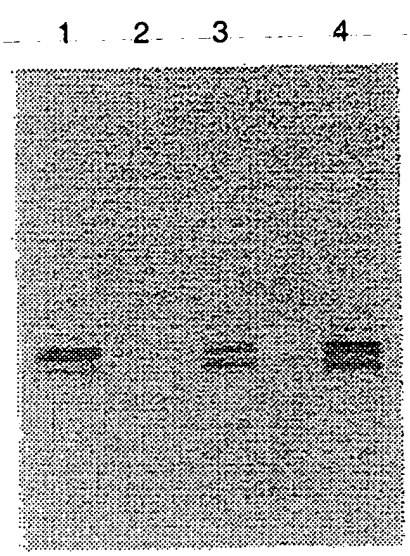

FIG._3A

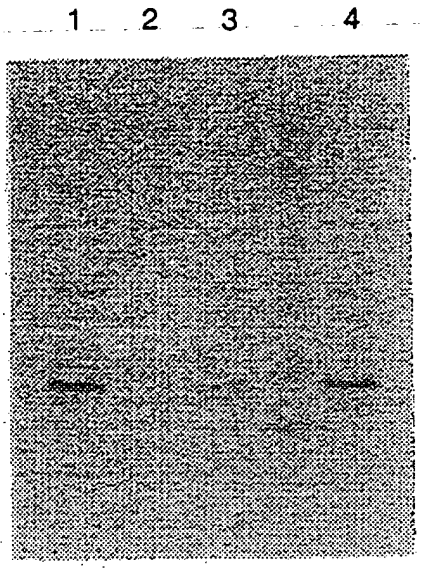

FIG._3B

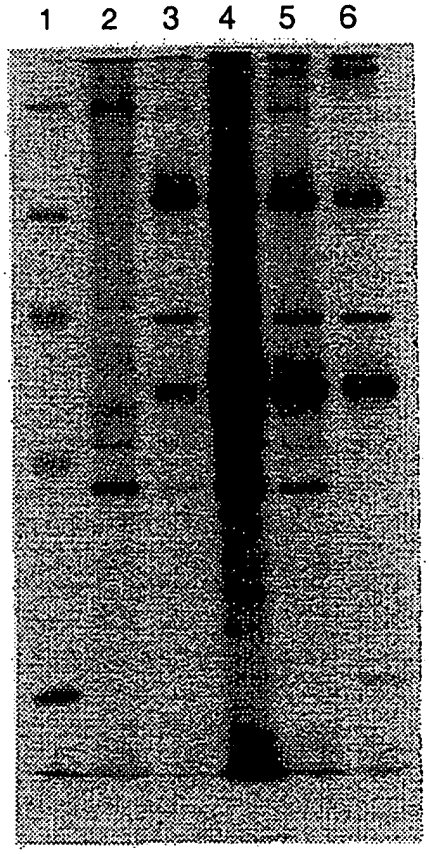

FIG._7

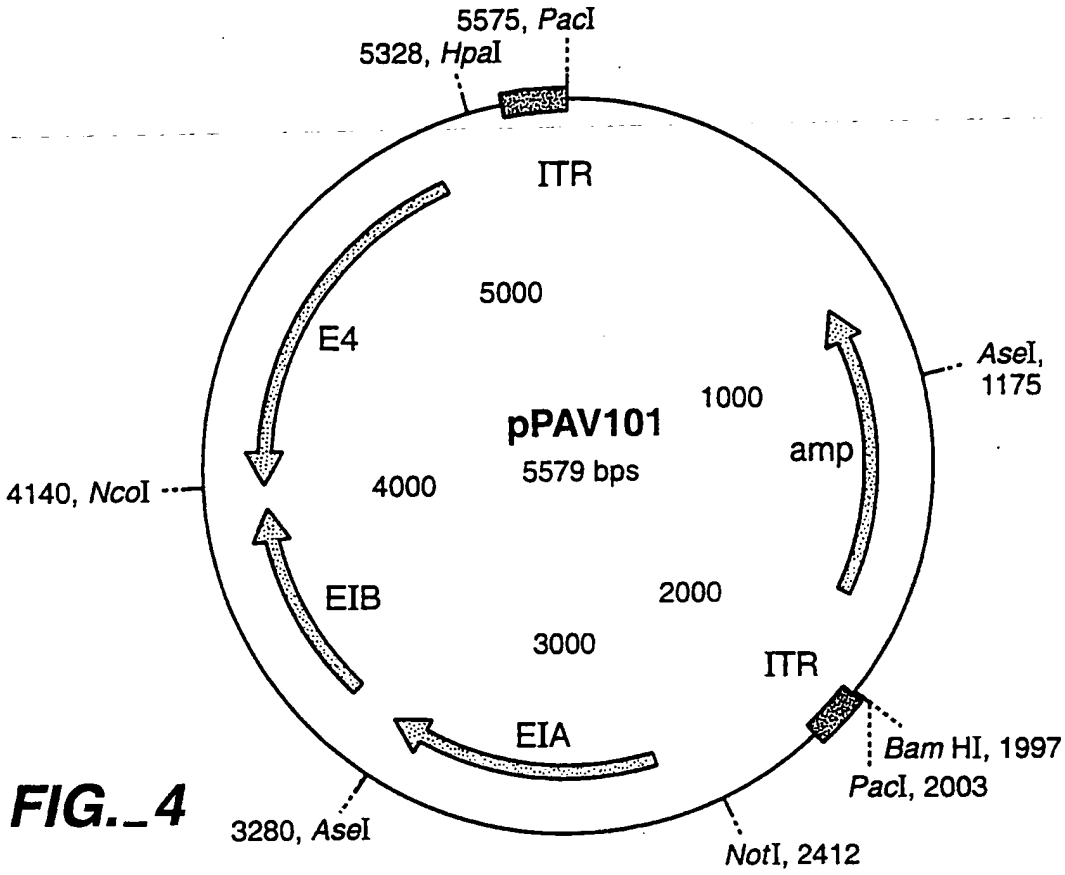
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FIG._4

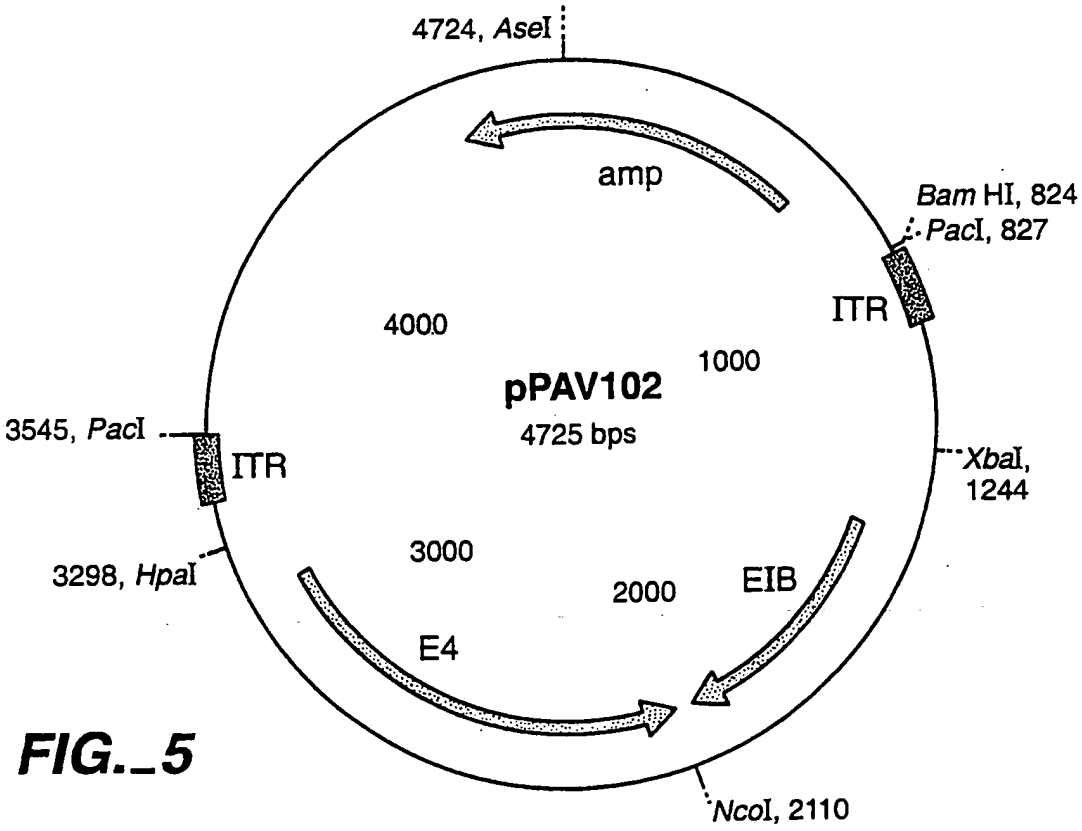
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FIG._5

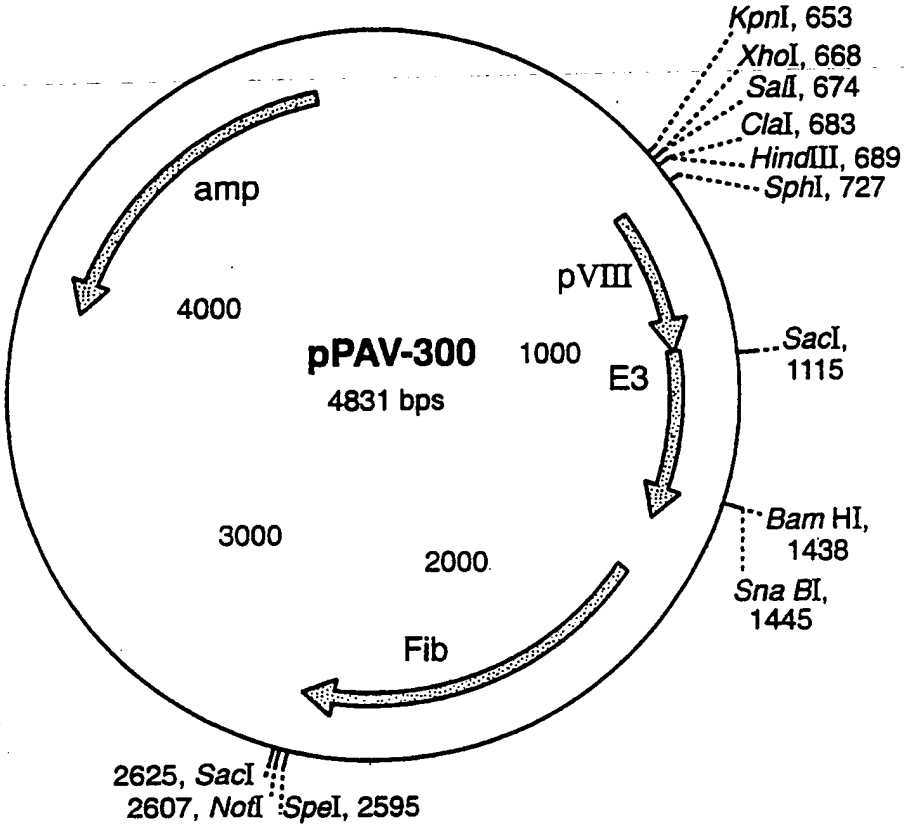
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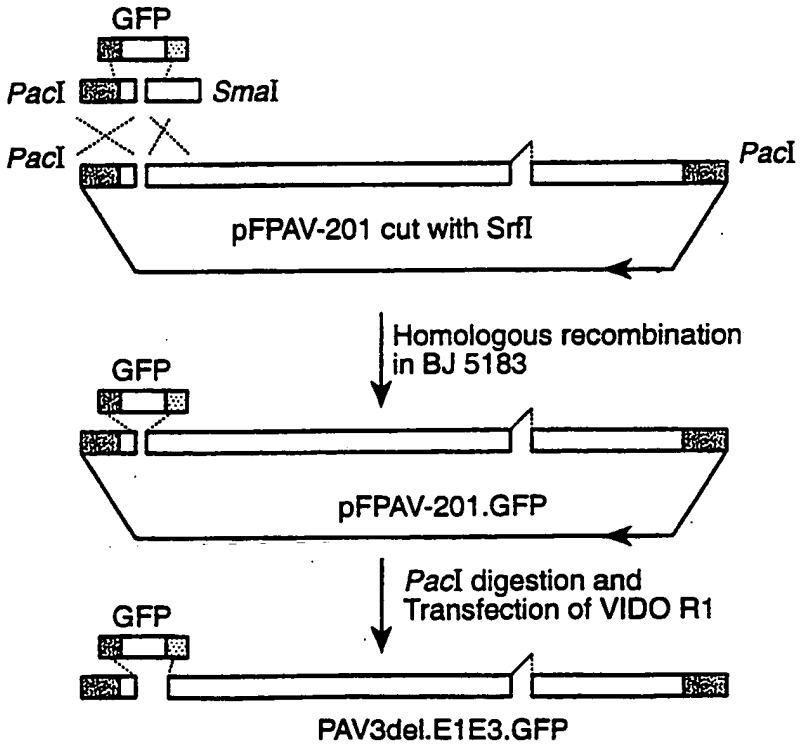
FIG._6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SmaI
PacI
PacI
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FIG._8

SEQUENCE LISTING

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Tikoo, Suresh
Babiuk, Lorne

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